The role of *Desulfitobacterium* spp. in the global network of *O*-demethylation in soil

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By Diplom-Umweltwissenschaftler Felix Sebastian Mingo

Born on 20.11.1988 in Reus (Spain)

First reviewer:	Prof. Dr. Gabriele Diekert	(Friedrich-Schiller-Universität Jena)
Second reviewer:	Prof. Dr. Dirk Hoffmeister	(Friedrich-Schiller-Universität Jena)
Third reviewer:	Prof. Dr. Harold L. Drake	(Universität Bayreuth)

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GLOSSARY

AE	Activating enzyme
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BLAST	Basic Local Alignment Search Tool
bp	Base pair
Cl-OHPA	3-chloro-4-hydrodxyphenylacetic acid
CoA	Coenzyme A
COG3894	Cluster of ortologous groups 3894
CH_3-FH_4	Methyltetrahydrofolate
CP	Corrinoid protein
DAPI	4'-6-Diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxyribonucleoside triphosphate
dTTP	Deoxythymidine triphosphate
Dsf	*Desulfitobacterium*
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	Ethylenediaminetetraacetic acid
FH_4	Tetrahydrofolate
FID	Flame ionization detector
FISH	Fluorescence *in situ* hybridization
FTHFS	Formyltetrahydrofolate synthetase
GC	Gas chromatography
gDNA	Genomic deoxyribonucleic acid
HPLC	High performance liquid chromatography
IMG	Integrated Microbial Genomes
Kbp	Kilo base pair
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Lysogeny broth

MCS	Multiple cloning site
MIQE	Minimum Information for Publication of Quantitative PCR Experiments
MT I	Mehtyltransferase I
MT II	Mehtyltransferase II
OD_{578}	Optical density measured at a wavelength of 578 nm
OHPA	OH-Phenylacetic acid (4-hydroxyphenylacetic acid)
OTU	Operational taxonomic unit
2,4,6-TCP	2,4,6-Trichlorophenol
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	*Para*-formaldehyde
pH	*Potentia hydrogenii*
qPCR	Quantitative polymerase chain reaction
RACE	Reductive activator of corrinoid-dependent enzymes
rdhA	Reductive dehalogenase subunit A
RNA	Ribonucleic acid
rpoB	RNA polymerase subunit beta
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SOC	Soil organic carbon
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
Tris	Tris-hydroxymethyl-aminomethane
v/v	Volume per volume
w/v	Weight per volume

SUMMARY

Bacteria that belong to the genus *Desulfitobacterium* are described to cleave the ether bond of lignin-borne phenyl methyl ethers via *O*-demethylation, an enzymatic reaction that consists in a series of methyl transfer reactions yielding methyltetrahydrofolate as an intermediate compound for energy conservation and biosyntheses, and a phenolic derivative of the growth substrate as end product. Phenyl methyl ethers occur naturally in soils and originate from lignin degradation by white rot fungi under aerobic conditions. In order to assess the possible involvement of the anaerobic desulfitobacteria in the network of lignin degradation in soils, their participation in the *O*-demethylation of phenyl methyl ethers in the environment was studied within the framework of this thesis.

The ability of several *Desulfitobacterium* species to *O*-demethylate the phenyl methyl ethers 4-hydroxyanisole, syringate, vanillate and isovanillate was studied in the presence of fumarate, nitrate, thiosulfate or Fe(III) as electron acceptor. Out of ten tested species, only *D. metallireducens* lacked the ability of *O*-demethylation. The number of putative demethylase operons in genomes of desulfitobacteria was highest in *D. hafniense* strains (up to 18 operons).

The participation of *Desulfitobacterium* spp. in the *O*-demethylation of phenyl methyl ethers in the environment was assessed in bacterial enrichment cultures that originated from five different topsoils sampled in the vicinity of Jena (Germany). The combination of the growth substrates syringate and thiosulfate triggered the enrichment of desulfitobacteria as confirmed via quantitative PCR detection of *Desulfitobacterium*-specific 16S rRNA genes. After their initial enrichment, a gradual loss of desulfitobacterial gene copies was observed. Based on community analyses via Illumina MiSeq technology, an outcompetion of desulfitobacteria by acetogens was suggested. To allow a better quantification of desulfitobacteria in environmental samples, a new qPCR assay was established that uses the *Desulfitobacterium*-specific formyltetrahydrofolate synthetase (FTHFS) gene as marker. In contrast to the 16S rRNA gene, of which several copies are present in each *Desulfitobacterium* genome, and that detects all gene copies per genome, the new FTHFS primer pair specifically recognizes only one copy.

In conclusion, *Desulfitobacterium* spp., which presumably couple the *O*-demethylation of phenyl methyl ethers to the reduction of nitrate, thiosulfate or Fe(III) in the environment, were demonstrated to rely on their methylotrophic metabolism within environmental consortia for survival. It is concluded that *Desulfitobacterium* spp. are involved in the *O*-demethylation of phenyl methyl ethers in the environment and play an important role within the network of lignin degradation in soils.

ZUSAMMENFASSUNG

Bakterien der Gattung *Desulfitobacterium* besitzen die Fähigkeit die Etherbindung in aus Lignin abstammenden Phenylmethylethern mittels *O*-Demethylierung zu spalten. Diese enzymatische Reaktion umfasst Methyltransfer-Reaktionen, bei denen einerseits Methyltetrahydrofolat als Intermediat des Energiestoffwechsels und für Biosynthesen entsteht, andererseits wird ein phenolisches Derivat des Wachstumssubstrates als Endprodukt gebildet. Phenylmethylether kommen natürlich in Böden vor und entstehen beim Ligninabbau durch Weißfäulepilze unter aeroben Bedingungen. Um die Bedeutung von anaeroben Desulfitobakterien im Netzwerk des Ligninabbaus in Böden einzuschätzen, wurde im Rahmen dieser Arbeit ihre Beteiligung an der *O*-Demethylierung von Phenylmethylethern in der Umwelt untersucht.

Die mögliche *O*-Demethylierung der Phenylmethylether 4-Hydroxyanisol, Syringat, Vanillat und Isovanillat wurde für mehrere *Desulfitobacterium*-Spezies in der Anwesenheit von Fumarat, Nitrat, Thiosulfat oder Fe(III) als Elektronenakzeptor untersucht. Von zehn getesteten Spezies war nur *D. metallireduccens* nicht zur *O*-Demethylierung befähigt. Die Anzahl an mutmaßlichen Demethylase-Operons in Genomen von Desulfitobakterien war in Stämmen der Spezies *D. hafniense* am höchsten (bis zu 18 Operons).

Die Beteiligung von *Desulfitobacterium* spp. an der *O*-Demethylierung von Phenylmethylethern in der Umwelt wurde in bakteriellen Anreicherungskulturen untersucht, die aus fünf Oberböden erzeugt wurden, die in der Umgebung von Jena (Deutschland) beprobt wurden. Die Kombination der Wachstumssubstrate Syringat und Thiosulfat begünstigte die Anreicherung von Desulfitobakterien, welche durch die Detektion *Desulfitobacterium*-spezifischer 16S rRNA-Genkopien mittels quantitativer PCR nachgewiesen wurde. Nach einer anfänglichen Anreicherung konnte ein Verlust an Genkopien festgestellt werden. Die Untersuchung der mikrobiellen Gemeinschaften mittels Illumina MiSeq-Technologie zeigte, dass eine Auskompetierung von Desulfitobakterien seitens acetogener Bakterien erfolgte. Um eine genauere Quantifizierung von Desulfitobakterien in Umweltproben zu ermöglichen, wurde ein qPCR-Assay basierend auf der Detektion des *Desulfitobacterium*-spezifischen Formyltetrahydrofolat Synthetase (FTHFS)-Gens etabliert. Im Gegensatz zum 16S rRNA-Gen, das mehrfach in jedem *Desulfitobacterium*-Genom vorhanden ist und somit mehrfach mittels qPCR detektiert wird, wird im FTHFS-Assay nur eine Genkopie pro Genom detektiert.

Zusammenfassend konnte gezeigt werden, dass *Desulfitobacterium* spp. in der Umwelt wahrscheinlich die *O*-Demethylierung von Phenylmethylethern an die Reduktion von Nitrat, Thiosulfat oder Fe(III) koppelt und den methylotrophen Stoffwechsel nutzt, um in mikrobiellen Konsortien zu überleben. *Desulfitobacterium* spp. sind demnach an der *O*-Demethylierung von Phenylmethylethern in der Umwelt beteiligt und spielen eine wichtige Rolle innerhalb des Netzwerkes des Ligninabbaus in Böden.

1 INTRODUCTION

1.1 *O*-demethylation of phenyl methyl ethers in the environment

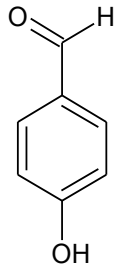
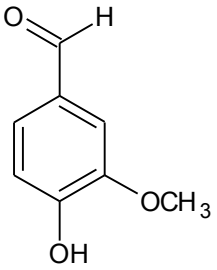
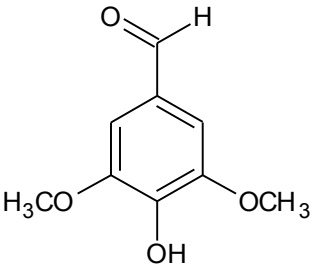
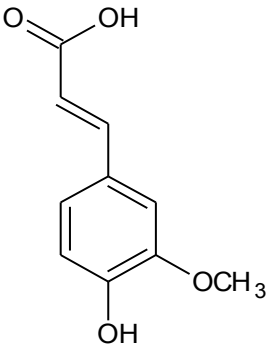
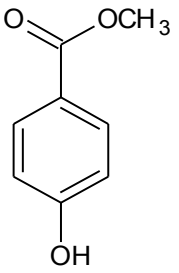
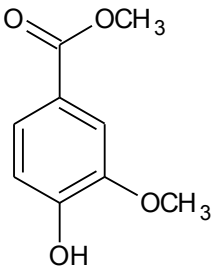
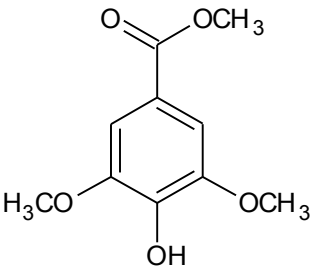
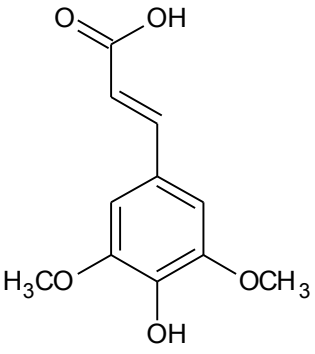
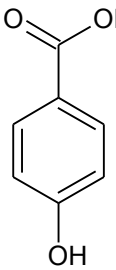
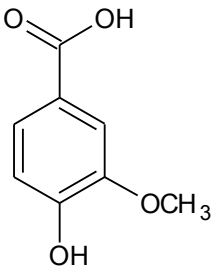
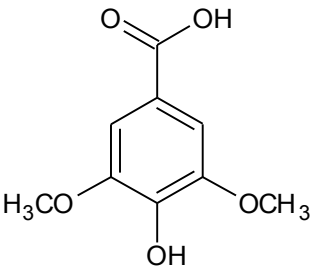
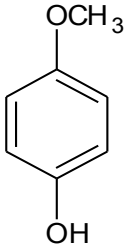
Lignin is the most abundant aromatic plant component in terrestrial ecosystems and, second to cellulose, the most abundant polymer found on Earth (Thevenot et al., 2010). As a major constituent of plant tissue, it represents a major carbon sink in the biosphere, accounting for about 30% of the carbon pool sequestered into plant material (approximately 1.4×10^{12} kg C a^{-1}) (Battle et al., 2000). Lignin can be described as a highly branched heteropolymer that associates with cellulose or hemicellulose in the cell wall of woody and non-woody plant tissues, providing strength and rigidity to the plant tissue (Barceló, 1997). It consists of hydroxylated and methoxylated phenylpropane monomeric units that are linked by a variety of C-C or C-O-C (e.g. alkyl-aryl ether) bonds (Kögel, 1986). Due to its complex structure, only few organisms are able to degrade this polymer. While brown rot basidiomycetes (Kirk & Adler, 1970; Filley et al., 2002) and bacteria of the genera *Nocardia* (Trojanowski et al., 1977) as well as *Streptomyces* (Antai & Crawford, 1981) are able to alter the structure of lignin, only white rot basidiomycetes are able to mineralize it (Boyle et al., 1992). The degradation of lignin in nature mainly occurs aerobically by the activity of extracellular enzymes such as lignin peroxidase, manganese peroxidase and laccase (Higuchi, 1990; ten Have & Teunissen, 2001; Sánchez, 2009). The reactions catalyzed by these enzymes lead to the release of the monomeric structural units from the lignin backbone into the environment, where they may be used as carbon and/or energy sources by bacterial communities. Among the different products of lignin decomposition, phenyl methyl ethers represent the methoxylated aromatic fraction.

1.1.1 Natural occurrence of phenyl methyl ethers

The composition of lignin with regards to its monomeric units can be backtracked to major plant groups. Differences have been described for gymnosperm (softwood) and angiosperm (hardwood) species. Gymnosperm lignin is principally composed of coniferyl alcohol-derived units and minor amounts of coumaryl and sinapyl alcohol-derived units (Adler, 1977), whereas angiosperm lignin contains similar amounts of coniferyl and sinapyl alcohol-derived units and small amounts of coumaryl alcohol-based units (Nimz, 1974). Syringyl and vanillyl units as well as their stronger branched ferulic and sinapic variations are common methoxylated structural units that are incorporated in similar quantities in the lignin backbone in both, gymnosperms and angiosperms (Hedges & Mann, 1979; Kögel, 1986). The structure of the

most abundant lignin monomeric units that can be found in the environment are shown in **Table 1.1**. Thus, different phenyl methyl ethers might be expected in soil depending on the vegetation, along with those compounds that are found in most woody and non-woody plant tissues (see **Table 1.1**). Moreover, it is known that softwood is enriched in lignin in comparison to hardwood (Rodríguez Couto & Sanromán, 2005). Hence, the quantity of phenyl methyl ethers available in soil may differ depending on the dominant vegetation form. Upon the lignolytic activity of lignin peroxidases, manganese peroxidases and laccases, phenyl methyl ethers may be transferred from the plant tissue to the soil via aboveground or belowground deposition of litter (Thevenot et al., 2010).

Table 1.1: Common phenolic lignin monomers released to the environment upon the activity of ligninolytic enzymes. The compound 4-hydroxyanisole is less common compared to the remaining compounds shown in this table, but was included because of its importance in this study. Adapted from Thevenot et al., 2010.

	p-Hydroxyl phenols	Vanillyl phenols	Syringyl phenols	Other phenols
Aldehydes	 *p*-Hydroxy-benzaldehyde	 Vanillin	 Syringaldehyde	 Ferulic acid
Ketones	 *p*-Hydroxy-acetophenone	 Acetovanillone	 Acetosyringone	 Sinapic acid
Acids	 *p*-Hydroxy-benzoic acid	 Vanillic acid	 Syringic acid	 4-Hydroxyanisole

Many of the compounds presented above can be detected in soils (Whitehead, 1964; Shindo et al., 1978; Baziramakenga et al., 1995) in quantities that can amount more than 1 mg g^{-1} soil organic carbon (SOC) (Katase, 1981). Hence, their use by microbial communities has been a main focus of interest to uncover a possible link between bacteria and fungi in the network of lignin degradation. Many studies reported on the consumption of syringic, vanillic and ferulic acids in microcosms derived from lake sediments (Kaiser & Hanselmann, 1982), forest soil (Küsel & Drake, 1995) or marsh sediments (Phelps & Young, 1996) under anaerobic conditions. In all studies, demethylated intermediates of the initial methoxylated aromatics accumulated in the media and were afterwards mineralized. Thus, it became clear that the first step in the anaerobic degradation of phenyl methyl ethers released from the lignin backbone is their *O*-demethylation to phenolic derivates.

1.1.2 Anaerobic *O*-demethylase systems

As mentioned above, it was shown earlier that many bacteria are specialized in the utilization of the methyl moiety of phenyl methyl ethers for energy conservation and growth. The demethylated substrate is usually secreted to the growth medium without further degradation. Since these bacteria feed solely on the methyl group of the methoxylated aromatic compound, they are called methylotrophs. The use of the methyl moiety is possible by cleaving the ether bond of the methoxylated aromatic substrate. The process of ether cleavage, known as *O*-demethylation, is catalyzed by tetrahydrofolate (FH_4)-dependent enzyme systems termed *O*-demethylases (Kaufmann et al., 1997). The gene expression of *O*-demethylases is typically induced by a specific substrate that can be cleaved by the enzyme system (Engelmann et al., 2001; Peng et al., 2011). The substrate is in most cases, but not necessarily, a growth substrate. *O*-demethylases consist of two methyltransferases (MT I and MT II), a corrinoid protein (CP) and an activating enzyme (AE) (Kaufmann et al., 1997; Schilhabel et al., 2009). **Figure 1.1** depicts the mechanism of the *O*-demethylase reaction.

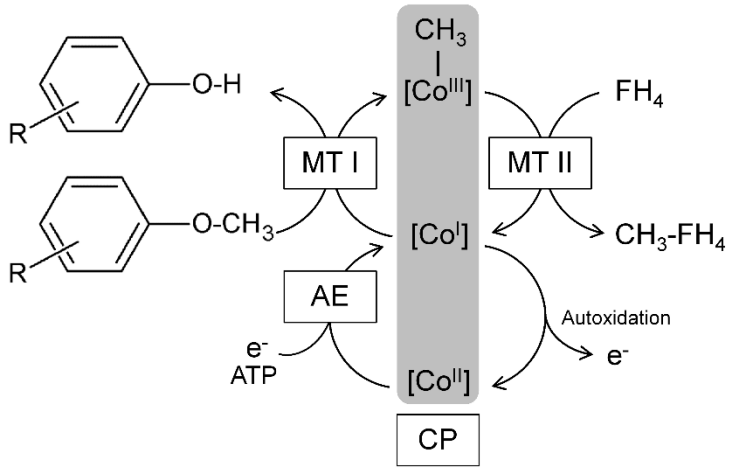


Figure 1.1: Scheme of the *O*-demethylase reaction adapted from Kaufmann et al. (1997). Abbreviations: AE (activating enzyme), [Co^{I-III}] (corrinoid protein [CP] with cobalt in the respective oxidation state), FH_4 (tetrahydrofolate), MT (methyltransferase).

In the first step, MT I, which is responsible for the substrate specificity of the *O*-demethylase (Engelmann et al., 2001; Schilhabel et al., 2009; Kreher et al., 2010), cleaves the ether bond of the methoxylated substrate and transfers the methyl group to the super-reduced corrinoid cofactor ([Co^{I}]) of CP, which acts as a methyl group carrier. Subsequently, MT II transfers the methyl group from the methylated corrinoid to FH_4 yielding methyltetrahydrofolate (CH_3-FH_4). CH_3-FH_4 is an intermediate in several downstream metabolic pathways and can be used for energy conservation (Drake et al., 2006), DNA methylation or amino acid synthesis (Sonoki et al., 2002). The last protein component of the system, AE, has a repair function after inadvertent oxidation of the super-reduced corrinoid cofactor ([Co^{I}]) to inactive [Co^{II}]-CP. It catalyzes the ATP-dependent electron transfer from an unknown electron donor to [Co^{II}]-CP yielding physiologically active [Co^{I}]-CP, allowing CP to participate in further *O*-demethylation reactions (Siebert et al., 2005; Sperfeld et al., 2014). From previous studies it is known that only one AE gene product is required for the reduction of the corrinoid proteins of different *O*-demethylases (Schilhabel et al., 2009; Studenik et al., 2012; Nguyen et al., 2013). In *Acetobacterium dehalogenans* and *Desulfitobacterium hafniense* DCB-2, the genes encoding for MT I, MT II and CP are usually organized into an operon or cluster, while the gene encoding for AE may be, but is not necessarily, located in the vicinity (Schilhabel et al., 2009; Studenik et al., 2012).

The *O*-demethylation of phenyl methyl ethers under anaerobic conditions was first described for acetogens (Naidu & Ragsdale, 2001), which are Firmicutes that use the Wood-Ljungdahl pathway to produce acetate from CO_2 and a variety of organic or inorganic C_1-precursors including phenyl methyl ethers (e.g. Bache & Pfenning, 1981; Daniel et al., 1991; Traunecker

et al., 1991; Stupperich & Konle, 1993; Liesack et al., 1994). The lack of reports on non-acetogenic Fimicutes capable of this process led for a long time to the assumption that acetogens were the only bacteria involved in the anaerobic degradation of methoxylated aromatic compounds in the environment. However, the reaction was later described for *Desulfitobacterium* spp. (Neumann et al., 2004), a non-acetogenic Firmicutes genus known for the reduction of sulphurous or chlorinated compounds. This turn of events raised the question which importance non-acetogenic Firmicutes might have in the global network of *O*-demethylation of phenyl methyl ethers in soils along with acetogenic bacteria.

1.1.3 Aerobic *O*-demethylation

Even though this thesis is centered on anaerobic *O*-demethylation of phenyl methyl ethers, the mechanisms of aerobic *O*-demethylation shall also be briefly described, as forest soils are mostly oxic environments and thus favor the growth of aerobic and facultative bacteria outside from anaerobic microniches. In microbes such as *Sphingomonas paucimobilis* SYK-6, aerobic *O*-demethylation may be mediated by NAD(P)H-dependent monooxygenases or FH_4-dependent methyltransferase systems, depending on the substrate. *S. paucimobilis* SYK-6 can demethylate 5,5'-dehydrovanillic acid under aerobic conditions via a monooxygenase system (Sonoki et al., 2000), while syringate and vanillate are demethylated via an aerobic FH_4-dependent *O*-demethylase system (Sonoki et al., 2002). The aerobic *O*-demethylation of vanillate via monooxygenases was also described for *Pseudomonas* spp. (Buswell & Ribbons, 1988), e.g. for *Pseudomonas* sp. strains ATCC19151 (Brunel & Davison, 1988) and HR199 (Priefert et al., 1997). The main difference between NAD(P)H-dependent monooxygenases and FH_4-dependent *O*-demethylases is that monooxygenase systems do not catalyze methyl transfer reactions. Instead, the demethylation is an oxidative process that proceeds via the formation of an unstable hemiacetal which spontaneously decomposes into an aldehyde and an alcohol (Dagley, 1986) (**Figure 1.2**). The aerobic FH_4-dependent *O*-demethylase system works as described above for the anaerobic *O*-demethylase reaction.

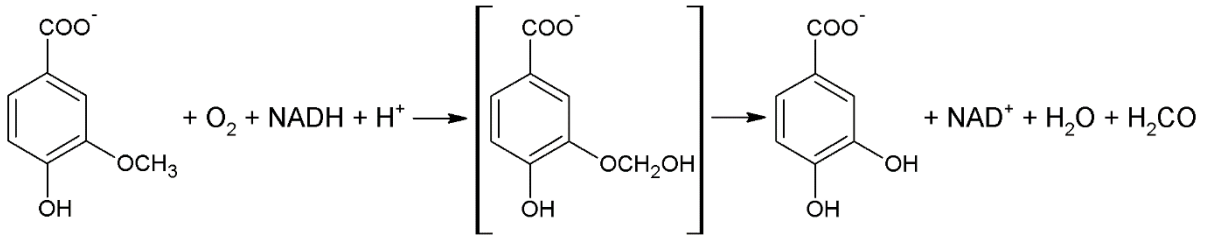


Figure 1.2: Oxidative *O*-demethylation of vanillate to protocatechuate via a hemiacetal intermediate, catalyzed by monooxygenases in *Pseudomonas* spp. (adapted from Brunel & Davison, 1988).

1.2 The *Desulfitobacterium* genus

Desulfitobacterium spp. are strictly anaerobic, rod-shaped, Gram-positive bacteria that belong to the phylum Firmicutes (Clostridia, Clostridiales, Peptococcaceae) (Spring & Rosenzweig, 2006). Most *Desulfitobacterium* species known so far were isolated from environments contaminated with halogenated compounds. The first isolated species of this genus was *Desulfitobacterium hafniense* DCB-2 (Madsen & Licht, 1992). However, the genus was not established until the isolation of *Desulfitobacterium dehalogenans* (Utkin et al., 1994). While being mostly known and studied for their ability to reductively dehalogenate natural and anthropogenic organohalide compounds (e.g. Dennie et al., 1998; Lanthier et al., 2005; Field & Sierra-Álvarez, 2008; Villemur, 2013), *Desulfitobacterium* spp. have a versatile metabolism that allows for them to use a wide range of electron donors (e.g. pyruvate, lactate, formate and hydrogen) and electron acceptors (e.g. nitrate, sulfurous compounds and metal cations) (Villemur et al., 2006). This distinguishes the metabolism of *Desulfitobacterium* spp. from the metabolism of obligate organohalide respirers such as *Dehalococcoides* spp. or *Dehalobacter* spp., which strictly depend on the process of reductive dehalogenation for energy conservation (Hug et al., 2013). The discovery of the methylotrophic metabolism of desulfitobacteria (Neumann et al., 2004) suggested that this genus, formerly thought to be mostly involved in the detoxification of organohalides, might also be involved in the network of bacterial lignin decomposition in soils along with acetogens. Furthermore, it hinted at a natural niche in uncontaminated habitats such as forest soil.

1.2.1 Methylotrophic metabolism of *Desulfitobacterium* spp. in comparison to acetogens

In acetogens and *Desulfitobacterium* spp., the methyl group in CH_3-FH_4, which is derived from the methoxylated growth substrate, is oxidized to CO_2 via the reverse methyl branch of the Wood-Ljungdahl pathway (Drake et al., 2006; **Figure 1.3 A**). In the case of acetogens, the reducing equivalents generated by methyl group oxidation are transferred to CO_2, which is reduced to carbon monoxide in a bound form and subsequently, along with additional methyl groups derived from CH_3-FH_4, converted to acetyl coenzyme A (acetyl-CoA) in the carbon monoxide dehydrogenase:acetyl-CoA synthase reaction (Drake et al., 2006). The resulting acetyl-CoA can then be converted to acetate via the reactions catalyzed by phosphate acetyltransferase and acetate kinase, yielding 0.75 mol ATP via substrate level phosphorylation per mol methyl group (**Figure 1.3 B**).

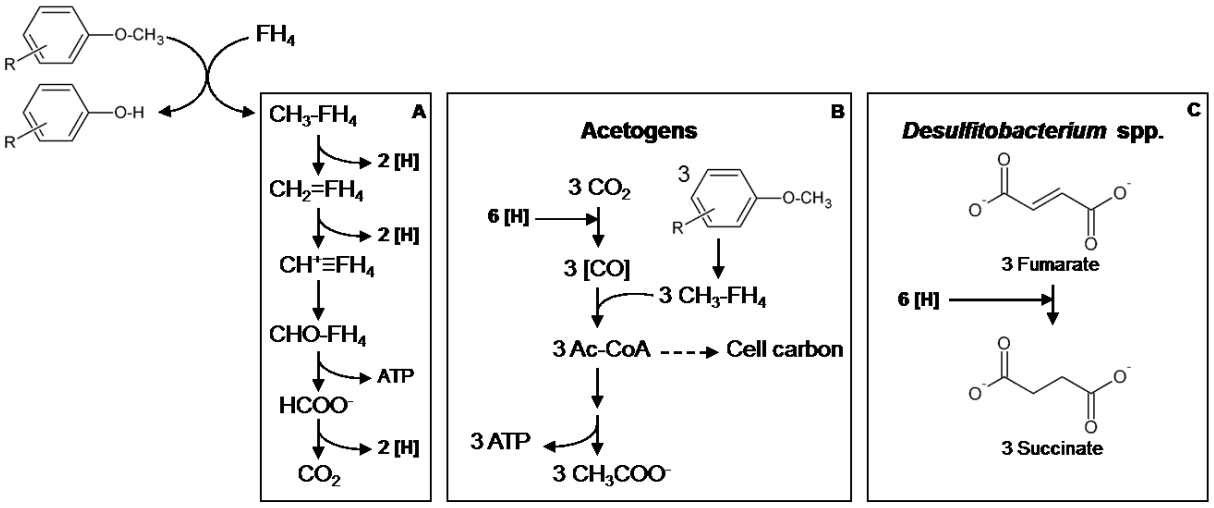


Figure 1.3: Comparison of the methylotrophic metabolism in acetogens and *Desulfitobacterium* spp. The methyl group in methyltetrahydrofolate is oxidized to CO_2 via the reverse methyl branch of the Wood-Ljungdahl pathway in both, acetogens and desulfitobacteria (A). The generated reducing equivalents are transferred to CO_2 in the case of acetogens (B). Since *Desulfitobacterium* spp. cannot use CO_2 as terminal electron acceptor, an alternate electron acceptor (for example fumarate) is needed as a sink for the reducing equivalents generated in the oxidation of the methyl group to CO_2 (C). Abbreviations: [H] (reducing equivalent), CH_3-FH_4 (methyltetrahydrofolate), CH_2=FH_4 (methylenetetrahydrofolate), CH^+≡FH_4 (methenyltetrahydrofolate), CHO-FH_4 (formyltetrahydrofolate).

In contrast to acetogens, *Desulfitobacterium* spp. were shown to be unable to use CO_2 as a terminal electron acceptor for *O*-demethylation (Neumann et al., 2004), even though all enzymes required for acetate synthesis from phenyl methyl ethers are present and active (Kreher et al., 2008). This unability might be due to the low activities of the phosphate acetyltransferase and/or carbon monoxide dehydrogenase. Aside from the ATP gained in the oxidation of the methyl group to CO_2, the Wood-Ljungdahl pathway is, in contrast to acetogens, rendered non-functional for energy conservation in desulfitobacteria. Instead, they are forced to rely on

alternate electron acceptors that might be present in soil or that may be provided by the soil microbial community (**Figure 1.3 C**). Additional energy can then be gained from the reduction of the corresponding electron acceptor. Thus, it was hypothesized that the utilization of the methyl group of methoxylated aromatic compounds in *Desulfitobacterium* spp. may have the purpose to channel methyl groups into anabolic pathways (Peng et al., 2011). In former studies it was shown that *D. hafniense* strains DCB-2 and PCE-S could couple the *O*-demethylation of vanillate to the reduction of fumarate to succinate and, in the case of *D. hafniense* DCB-2, also of 3-chloro-4-hydroxyphenylacetic acid (Cl-OHPA) to 4-hydroxyphenylacetic acid (OHPA) (Neumann et al., 2004). However, nothing is known about physiological electron acceptors that might be present in natural environments such as forest soil, where a variety of different acceptors might be available for the *O*-demethylation of phenyl methyl ethers.

The recent characterization of an *O*-demethylase from *D. hafniense* DCB-2 (locus tags Dhaf_4610, 4611, 4612) allowed to gain further insights into the methylotrophic metabolism of the *Desulfitobacterium* genus (Studenik et al., 2012). The purified MT I showed its highest activity for the phenyl methyl ether guaiacol and lower activities for syringate and vanillate. Furthermore, several putative demethylase systems could be identified in the genome of this organism. Thus, it was hypothesized that *D. hafniense* DCB-2 might be able to utilize various phenyl methyl ethers for growth, and that the methylotrophic metabolism might play an important role for the generation of reducing equivalents, which may then be used for the reduction of organic or inorganic electron acceptors.

1.2.2 The presence of *Desulfitobacterium* spp. in the environment

Since *Desulfitobacterium* spp. are mainly studied for their ability to reductively dehalogenate natural and anthropogenic halogenated organic compounds, the presence of this genus has mostly been reported for sites polluted with such compounds. **Table 1.2** provides an overview of the sources of *Desulfitobacterium* spp. isolated so far.

Table 1.2: *Desulfitobacterium* species isolated from different habitats. Abbreviations: PCE (tetrachloroethene), TCE (trichloroethene), 1,2-DCA (1,2-dichloroethane). Adapted from Villemur et al. (2006).

Species	Isolated from	Reference
D. hafniense DCB-2	Municipal sludge	Madsen & Licht (1992)
D. dehalogenans	Freshwater sediment	Utkin et al. (1994)
D. chlororespirans	Compost soil	Sanford et al. (1996)
D. hafniense PCP-1	Sewage sludge	Bouchard et al. (1996)
Desulfitobacterium sp. PCE1	PCE-contaminated soil	Gerritse et al. (1996)
D. hafniense PCE-S	PCE-contaminated soil	Miller et al. (1997)
D. hafniense TCE1	TCE-contaminated soil	Gerritse et al. (1999)
D. hafniense DP7	Human faeces	Van de Pas et al. (2001)
D. hafniense GBFH	River sediment	Niggenmyer et al. (2001)
D. hafniense TCP-A	River sediment	Breitenstein et al. (2001)
D. hafniense Y51	PCE-contaminated soil	Suyama et al. (2001)
D. metallireducens	River sediment	Finneran et al. (2002)
D. dichloroeliminans	1,2-DCA-contaminated soil	De Wildeman et al. (2003)
D. hafniense G2	Soil	Shelobolina et al. (2003)
Desulfitobacterium sp. KBC1	Soil	Tsukagoshi et al. (2006)

Most of the habitats presented above had an historical record of contamination (see corresponding references). However, the presence of desulfitobacteria has also been reported in environments not contaminated with halogenated compounds (Lanthier et al., 2001). It was suggested that the genus *Desulfitobacterium* might be ubiquitously distributed in soils. Another recent study reported the presence of desulfitobacteria in Tinto River in Spain, which represents a natural acidic rock drainage environment (Sánchez-Andrea et al., 2011). Here, *Desulfitobacterium* spp. seemed to play a role in the cycling of metals. Desulfitobacteria could also be detected in various US lake sediments that represented a geographical sulfur gradient, suggesting their participation in the sulfur cycle (Krzmarzick et al., 2013). Last, but not least, it was shown that the presence of *Desulfitobacterium* spp. in soils positively correlated with increasing soil compaction (Hartmann et al., 2014). From the studies presented above it can be concluded that the presence of *Desulfitobacterium* spp. is not restricted to habitats with a contamination record. Their suggested ubiquity in the environment can be attributed to their versatile metabolism, which features the ability to utilize various compounds as electron acceptors under anaerobic conditions, such as sulfurous compounds and metal cations.

However, so far no study has focussed on the influence of the electron-donating compound on their presence in the environment. As phenyl methyl ethers are widespread in the environment, the *O*-demethylation of these lignin degradation products may be coupled to the reduction of the above mentioned electron acceptors. This is feasible in forest soils, where a variety of electron acceptors is provided from both the weathering of bedrock and from the mineralization of soil organic matter, and where phenyl methyl ethers are most likely an abundant carbon and/or energy source.

1.3 Aims of the study

So far, research on the methylotrophic metabolism of *Desulfitobacterium* spp. has focussed on the elucidation of the involved pathways (Neumann et al., 2004) and the biochemical characterization of associated enzymes (Kreher et al., 2008; Studenik et al., 2012). These lab-scale studies helped to understand how desulfitobacteria might be involved in the *O*-demethylation of phenyl methyl ethers in the environment. However, previous research was only centered on *D. hafniense* strains DCB-2 and PCE-S, and no knowledge existed on the *O*-demethylation potential of other *D. hafniense* strains or other *Desulfitobacterium* species. Moreover, no physiological electron acceptor possibly present in the environment had been identified so far, raising the question if *O*-demethylation is an option for these organisms to thrive in natural habitats. Therefore, one goal of this study was to elucidate whether *O*-demethylation is a common metabolic feature of the genus *Desulfitobacterium* and which naturally occurring electron acceptors may be utilized by these microbes. This was assessed in growth experiments by coupling the *O*-demethylation of different phenyl methyl ethers to the reduction of electron acceptors that occur in soil. In order to evaluate the importance of methylotrophic metabolism for this genus, the genetic background of *O*-demethylation was investigated in *Desulfitobacterium* genomes that have been published so far, following the identification strategy presented in the study of Studenik et al. (2012).

Previous studies lacked the confirmation that the knowledge gained from laboratory experiments is applicable to experiments that mimic environmental conditions. Therefore, another aim was to demonstrate that desulfitobacteria are involved in *O*-demethylation processes in soils. For this, five different soils were sampled in the vicinity of Jena (Germany), and an enrichment of desulfitobacteria was attempted by using the *O*-demethylation of a phenyl methyl ether as the growth-selective process. The detection of *Desulfitobacterium* spp. was then conducted via fluoresecence *in situ* hybridization (FISH) and quantitative PCR (qPCR), using

two marker genes (16S rRNA and formyltetrahydrofolate synthetase genes). Moreover, the enriched microbial consortia were analyzed via Illumina MiSeq technology to unravel methylotrophic anaerobes that may be obtained from forest and grassland topsoil.

2 MATERIALS AND METHODS

2.1 Materials and microorganisms

All chemicals and reagents in this study, if not stated otherwise, were purchased at the highest available purity from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany), AppliChem GmbH (Darmstadt, Germany), Merck KGaA (Darmstadt, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and VWR International GmbH (Darmstadt, Germany). All gases were obtained from Linde AG (Leuna, Germany).

Desulfitobacterium hafniense strains DCB-2 (DSM-10664), DP7 (DSM-13498), G2 (DSM-16228), PCE-S (DSM-14645), PCP-1 (DSM-12420), and TCP-A (DSM-13557) as well as *D. chlororespirans* (DSM-11544), *D. dehalogenans* (DSM-9161), and *D. metallireducens* (DSM-15288) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *D. hafniense* strain Y51 was taken from the strain collection of the Department of Applied and Ecological Microbiology of the Friedrich Schiller University Jena and was kindly provided by Taiki Futagami (Kagoshima University, Kagoshima, Japan).

Escherichia coli strain XL1 blue was also taken from the strain collection.

2.2 Microbiological methods

2.2.1 Cultivation of *Desulfitobacterium* spp.

Desulfitobacterium spp. were cultivated under anoxic conditions. For *D. chlororespirans*, *D. dehalogenans* and *D. hafniense* strains, the growth medium was composed of basal medium, yeast extract (0.2% w/v) and resazurin (1 mg/l) according to Neumann et al. (2004). For *D. metallireducens*, modified basal medium was used (Finneran et al., 2002) (**Table 2.1**).

Table 2.1: Composition of the basal medium used for the cultivation of *Desulfitobacterium* spp. All quantities are expressed in gram per liter (g/l).

Component	*Desulfitobacterium* spp.	*D. metallireducens*
Na_2SO_4	0.07	-
KH_2PO_4	0.20	0.60
NH_4Cl	0.25	0.30
NaCl	1.00	-
$MgCl_2 \times 6\ H_2O$	0.40	-
$MgSO_4 \times 7\ H_2O$	-	0.50
KCl	0.50	-
$CaCl_2 \times 2\ H_2O$	0.15	0.10
Fe(III) citrate	-	10.0

The media were poured into 50, 100 or 200 ml-serum flasks, filling only half of the flask's volume, and sealed with rubber stoppers and aluminum caps. Then, all bottles were flushed with N_2 and evacuated at least 25 times (2.5 min each cycle) and autoclaved. Afterwards, the media were completed by addition of 4 ml 1 M $NaHCO_3$, 1 ml supplement solution (see **Table 2.2** for composition), 0.25 ml 36 mM $FeSO_4$ (dissolved in 50 mM H_2SO_4) and 0.1 ml 5% (w/v) cysteine-HCl to 100 ml medium. The pH of the growth medium ranged from 7.5–8.0. Prior to inoculation of the media, electron donors (pyruvate, 40 mM; syringate, vanillate, isovanillate, 4-hydroxyanisole, 2 mM) and electron acceptors (fumarate in combination with pyruvate or in combination with phenyl methyl ethers, 40 mM or 15 mM, respectively; nitrate, 10 mM; thiosulfate, 5 mM; Fe(III) citrate, 12 mM) were added from separately autoclaved anoxic stock solutions. The media were inoculated with 10% (v/v) of a pre-culture grown with 40 mM pyruvate and 40 mM fumarate in the case of *D. chlororespirans*, *D. dehalogenans* and *D. hafniense*. For *D. metallireducens* the pre-culture was grown with 40 mM pyruvate and 38 mM Fe(III) citrate. The cultivation took place in a water bath shaker at a temperature of 28°C and 150 rpm. The growth of the cultures was followed either by measuring the optical density at a wavelength of 578 nm (OD_{578}) or by determination of the protein concentration after alkaline cell lysis using the method of Bradford (1976) (see 2.3.1). Samples were taken periodically for the measurement of substrate concentrations (see 2.3.2). The maintenance of *Desulfitobacterium* spp. and strains took place with 40 mM pyruvate and 40 mM fumarate with the exception of *D. metallireducens* (40 mM pyruvate and 38 mM Fe(III) citrate).

Table 2.2: Composition of the supplement solution.

Solution	Volume	Components	Content l^{-1}
Solution A – Trace metals	6 ml	HCl 25%	50 ml
		$FeSO_4 \times 7\ H_2O$	5000 mg
		$ZnCl_2$	350 mg
		$MnCl_2 \times 4\ H_2O$	500 mg
		H_3BO_4	30 mg
		$CoCl_2 \times 6\ H_2O$	950 mg
		$CuCl_2 \times 2\ H_2O$	10 mg
		$NiCl_2 \times 6\ H_2O$	120 mg
		$Na_2MoO_4 \times 2\ H_2O$	180 mg
Solution B – Vitamins	3 ml	*p*-Aminobenzoate	400 mg
		D(+) biotin	100 mg
		Nicotinic acid	1000 mg
		Ca-D(+) pantothenate	500 mg
		Pyridoxamine-HCl	1500 mg
		Thiamine-HCl	1000 mg
Solution C – Vitamin B_{12}	6 ml	Vitamin B_{12}	1000 mg
Solution D – Selenite	0.6 ml	$Na_2SeO_3 \times 5\ H_2O$	130 mg
Solution E – Tungsten	1.2 ml	$Na_2WO_4 \times 2\ H_2O$	165 mg
Potassium phosphate buffer	30 ml	KH_2PO_4	25.31 g
		K_2HPO_4	141.78 g
Ultra-pure water	253 ml		

2.2.2 Cultivation of *Escherichia coli* strain XL1 blue

E. coli XL1 blue cells were thawed upon retrieval from the -80°C freezer and were cultivated in liquid or solid lysogeny broth (LB) medium (5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl; in case of solid medium addition of 15 g/l Agar Kobe I). Cultures were incubated at 28°C (liquid media) or 37°C (solid media). In the case of transformation of *E. coli* XL1 blue with cloning vectors, the media additionally contained 100 µg/ml ampicillin for antibiotic selection of transformants.

2.2.3 Inoculation and cultivation of bacterial enrichment cultures

Bacterial enrichment cultures were derived from forest and grassland topsoils collected in Stadtroda (cambisol; 50°51'57.12"N, 11°43'0.11"E; deciduous forest), Tautenburg (luvisol; 50°59'33.64"N, 11°42'39.97"E; deciduous forest), Bad Klosterlausnitz (gleysol; 50°54'54.18"N, 11°51'20.83"E; coniferous forest), Jena (pelosol; 50°55'50.11"N, 11°37'39.28"E; non-arable grassland) and Trockenborn-Wolfersdorf (podsol; 50°47'34.78"N, 11°39'42.16"E; coniferous forest), respectively. The approximate locations are shown in **Figure 2.1**. All soils were sampled with a topsoil sampler and frozen at -20°C upon use.

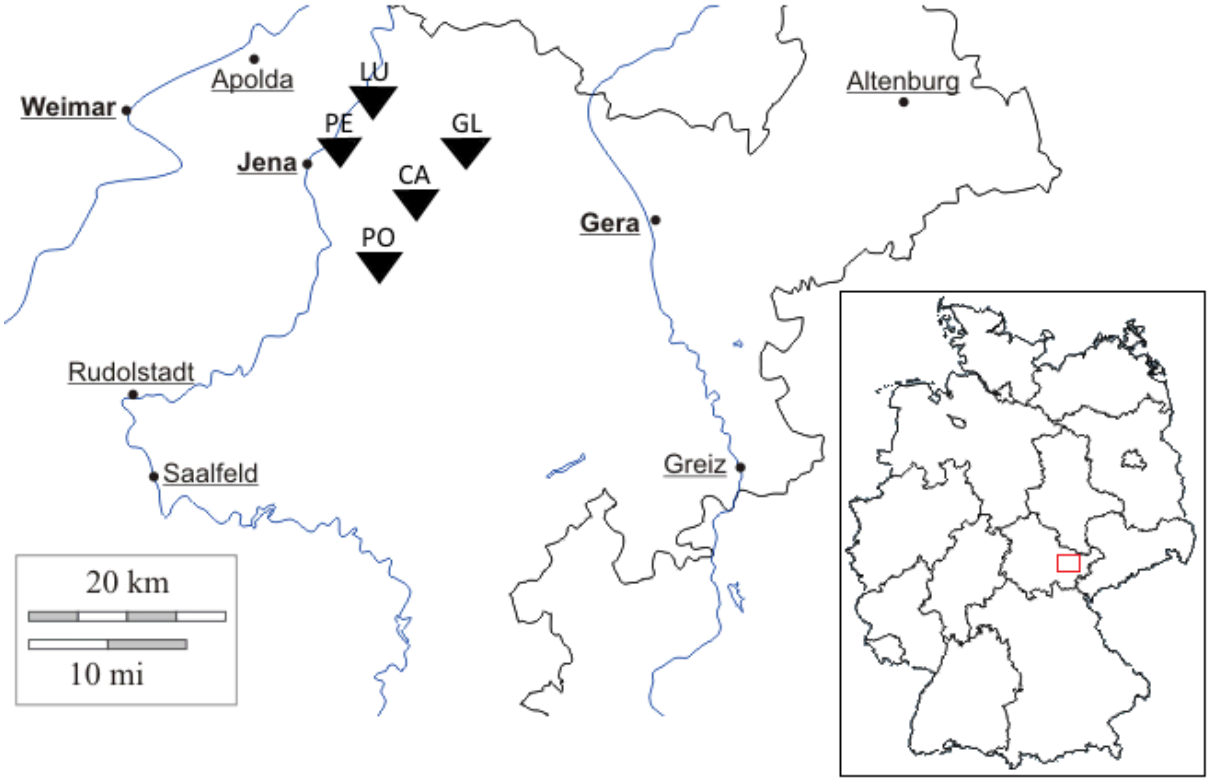


Figure 2.1: Approximate locations of the soil sampling sites in the Federal State of Thuringia, Germany. The red square in the miniature map of Germany shows the overall location of the sampling sites in Germany. Abbreviations: CA (cambisol), LU (luvisol), GL (gleysol), PE (pelosol) and PO (podsol).

For the enrichment of *O*-demethylating bacteria, 5 g bulk topsoil (fresh weight) were weighed into sterile 100 ml-serum flasks. The bottles were subsequently sealed with rubber stoppers and aluminum caps. They were evacuated and flushed with N_2 (100%) to ensure anoxic culturing conditions (20 cycles, 2.5 min each). Then, 45 ml of anoxic growth medium according to Neumann et al. (2004) (see 2.2.1), but containing only 0.02% (w/v) yeast extract, were added via a syringe. The electron donor (syringate) and electron acceptors (thiosulfate, nitrate, 3-chloro-4-hydroxyphenylacetic acid and 2,4,6-trichlorophenol) were supplied from separately autoclaved anoxic stock solutions. The concentrations applied were as follows: syringate, 4

mM, and when combined with 2,4,6-trichlorophenol, 0.5 mM; thiosulfate, 4 mM; nitrate, 5 mM; 3-chloro-4-hydroxyphenylacetic acid (Cl-OHPA), 5 mM; 2,4,6-trichlorophenol (2,4,6-TCP), 80 µM. Cultures were incubated in a water bath shaker at 28°C and 150 rpm. Samples were taken periodically for the measurement of substrate and protein concentrations (see 2.3). Once syringate could no longer be detected via HPLC, 5 ml of the enrichment cultures were transferred to 50 ml of fresh medium. All enrichments with the electron donor/acceptor couple syringate/thiosulfate consisted of biological duplicates and were transferred five times, except for podsol enrichment cultures, which were transferred three times due to low conversion rates. Cambisol enrichments with electron acceptors different from thiosulfate were unique replicates.

2.2.4 Storage of bacterial cultures

Short-term conservation of cultures took place at 4°C. Long-term conservation took place in anoxic 10 ml flasks containing 550 µl of a mixture composed of glycerol (85% v/v), cysteine-HCl (0.05% w/v) and Na_2S (0.05% w/v), sealed with rubber stoppers and aluminum caps. For conservation, 5 ml of bacterial culture were added to these flasks via a syringe. The samples were subsequently mixed and frozen at -80°C.

2.3 Analytical methods

Bacterial cultures were analyzed with regards to growth and substrate concentrations by taking a 1 ml sample out of growing cultures. The samples were centrifuged for 1 min at 16,000 ×g. For determination of the substrate concentrations, the supernatant of samples was analyzed. For the determination of bacterial growth, the cell pellet was used instead.

2.3.1 Determination of growth

Optical density

The optical density of *Desulfitobacterium* spp. was measured in transparent plastic cuvettes at a wavelength of 578 nm after addition of a small quantity of sodium dithionite to a 1 ml culture sample. If the OD_{578} of the sample was higher than 0.3, the sample was diluted appropriately. The measurement took place in a Spectrophotometer (Pharmacia Biotech Novaspec II, Uppsala, Sweden). Distilled water was used as sample blank.

Protein concentration

The protein concentration was used as an indicator for bacterial growth and was determined by the Bradford method (Bradford, 1976) after alkaline lysis of the cells. For this, a 1 ml culture pellet was re-suspended in 200 µl distilled water at an OD_{578} value of 0.2. For each additional OD of 0.2, 200 µl additional water were added. Then, 50 µl of 0.5 M NaOH were added per each 200 µl suspension, followed by an incubation of 5 min at 95°C. Samples were then centrifuged for 1 min at 16,000 ×g and 50 µl of each sample's supernatant were pipetted in triplicate into the wells of a microtiter plate. Subsequently, 200 µl of 1:5 diluted reagent (Roti-Nanoquant, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were added to each well. The absorption was read against a BSA calibration curve (0–100 µg/ml) at wavelengths of 450 and 590 nm in a VERSAmax tunable microplate reader (Molecular Devices, Biberach an der Riss, Germany).

2.3.2 Quantification of substrates and products

High performance liquid chromatography (HPLC)

High performance liquid chromatography was used to monitor the concentrations of phenyl methyl ethers, their demethylated products and of chlorinated phenols. Samples were diluted twentyfold in ultrapure water in a final volume of 1 ml. A LiChrospher 100 RP-8 125 × 4 mm column (Merck KGaA, Darmstadt, Germany) was used for separation with 25% (v/v) methanol and 0.3% (v/v) H_3PO_4 in water as eluent. The flow rate applied was 0.4 ml/min. Signals were detected at 210 nm. Under these conditions, the retention times were as follows: fumarate, 4.8 min; gallate, 4.9 min; protocatechuate, 6.4 min; vanillate, 8.3 min; 4-hydroxyanisole, 8.6 min; isovanillate, 8.8 min; syringate, 9.2 min; Cl-OHPA, 19.0 min. For separation of fumarate, vanillate, and its demethylated end product protocatechuate, the following gradient with a flow rate of 0.3 ml/min was applied: 0-11 min, 0.3% (v/v) H_3PO_4; 11-21 min, 0-25% (v/v) methanol plus 0.3% (v/v) H_3PO_4; 21-30 min, 25% (v/v) methanol plus 0.3% (v/v) H_3PO_4. Under these conditions, the retention times were as follows: fumarate, 7.1 min; protocatechuate, 14.9 min; vanillate, 24.0 min. All peak areas were read against a calibration curve of the corresponding compound.

Gas chromatography (GC)

Gas chromatography was used to measure the concentration of dissolved acetate in the growth medium of enrichment cultures. For this, 95 µl of sample were mixed with 5 µl pure formic

acid. The separation of organic acids took place in a Shimadzu Gas Chromatograph GC-14A (Shimadzu Europa GmbH, Duisburg, Germany) using an OPTIMA WAX 25 m × 32 mm (0.25 µm) column (Macherey-Nagel, Düren, Germany) and N_2 as the carrier gas (mobile phase). Before measurement, the oven, injector and detector chambers of the chromatograph were heated to 80°C, 150°C and 250°C, respectively. The flame ionization detector (FID) was started by igniting an H_2 flame with a lighter. Two microliters of a liquid sample were injected with a glass syringe and organic acids were separated according to the following temperature program: 80°C for 2 min, followed by an increase in temperature of 25°C per minute up to 200°C and holding the temperature of 200°C for 5 min. Under these conditions, acetate and formate had a retention time of approximately 5 and 5.4 min, respectively. All peak areas were read against a calibration curve of 0–15 mM acetate (formate was not quantified).

Detection of nitrite and nitrate

Nitrite was measured by the diazotization reaction described by Lunge (1904). The colorimetric reaction was started by the addition of 50 µl of 1% (w/v) sulphanilic acid and 50 µl of 0.3% (w/v) 1-naphthylamine to 50 µl of the diluted sample. After 5 min incubation at room temperature, absorption was measured in triplicates at a wavelength of 525 nm against a $NaNO_2$ calibration curve (0–200 µmol/l) in a VERSAmax tunable microplate reader.

For nitrate determination, the method of Bosch Serrat (1998) was followed. Diluted samples were mixed in a 1:1 ratio with 200 µl of a chloride solution consisting of 0.28 M NaCl dissolved in 9.35% (v/v) H_3PO_4. Subsequently, 1 ml of 0.55% (w/v) resorcinol dissolved in 62.5% (v/v) H_2SO_4 was added to each sample. The mixtures were incubated at 95°C for 8 minutes. They were allowed to cool down for 20 minutes and absorbance was measured in triplicates at 505 nm against a $NaNO_3$ calibration curve (0–1 mmol/l). Since in this assay both, nitrite and nitrate were detected, nitrate concentration was calculated by subtracting the nitrite concentration from the obtained value.

Detection of thiosulfate

Thiosulfate was measured according to Quentin & Pachmayr (1964). Diluted samples were mixed in a 3:1 ratio with 0.006% (w/v) methylene blue in 6 M HCl. After 3 h of incubation at room temperature, the absorbance was measured in triplicates at 660 nm against a $Na_2S_2O_3$ calibration curve (0–80 µmol/l) in a VERSAmax tunable microplate reader.

Detection of Fe(II), Fe(III) and total iron

The measurement of soluble Fe(II) and total iron was done according to the protocol of Viollier et al. (2000). For the determination of Fe(II), 1 ml of diluted sample was mixed with 100 µl of 0.01 M ferrozine dissolved in 0.1 M NH_4CH_3COO. The addition of ferrozine leads to the complexation of Fe(II) in the sample, which can then be detected. Subsequently, 150 µl of the samples were pipetted in triplicates to the wells of a microtiter plate for the determination of the Fe(II) concentration, but the absorbance was not yet measured until after the following steps. The total iron content was determined with the remaining volume of the same samples. For this, samples (650 µl) were mixed with 122 µl of 1.4 M hydroxylamine hydrochloride dissolved in 2 M HCl and incubated for 10 minutes at room temperature for the reduction of Fe(III) to Fe(II). Finally, 41 µl of a 10 M NH_4CH_3COO (pH 9.5) solution were added to the samples. In this last step, the Fe(II) derived from the reduction of Fe(III) forms a complex with the ferrozine that was initially added to each sample. Again, 150 µl of samples were pipetted in triplicates to the wells of a microtiter plate. The absorption of all samples before and after the reduction step with hydroxylamine hydrochloride was measured at 562 nm against a $FeCl_3$ calibration curve (0–80 µmol/l). The values obtained before the addition of the hydroxylamine hydrochloride and 10 M NH_4CH_3COO solution corresponded to the solube Fe(II) fraction, whereas the values obtained after the addition of these solutions corresponded to the total iron fraction in the medium. The Fe(III) concentration was obtained by substracting the Fe(II) concentration from the total iron concentration.

2.3.3 pH measurement of topsoils

The pH measurement of soils is based on the exchange between H^+ ions adsorbed to the surface of mineral and organic soil colloids and the ions in an aqueous solution (Blume et al., 2010). 10 g of bulk topsoil (fresh weight) were suspended in 25 ml 10 mM $CaCl_2$ and stirred at room temperature for two hours. Afterwards, the pH value was measured with a pH electrode.

2.4 Molecular biology methods

2.4.1 Isolation of genomic DNA

For the isolation of genomic DNA from *Desulfitobacterium* spp., a 1 ml sample of a grown culture ($OD_{578} \approx 0.6$) was taken and centrifuged at 16,000 ×g for 1 min. The supernatant was removed and DNA was extracted from the cell pellet using the innuPREP Bacteria DNA Kit (Analytic Jena AG, Jena, Germany) according to the manufacturer's instructions.

For the isolation of genomic DNA from soil, the Power Soil DNA Isolation Kit (Dianova GmbH, Hamburg, Germany) was used following the recommended procedure by the manufacturer. For this, 250 mg of bulk soil (fresh weight) were used.

The isolation of genomic DNA from bacterial enrichment cultures took place after the electron donor (syringate) could no longer be detected via HPLC. The centrifugation (16,000 ×g, 1 min) of a 2 ml sample was followed by the removal of the supernatant. For the first sub-cultivation step of every enrichment, the Power Soil DNA Isolation Kit was used. For all the remaining sub-cultivation steps, the innuPREP Bacteria DNA Kit was used.

The extracted DNA was quantified using a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Schwerte, Germany) and stored for short term at 4°C or for long term at -20°C.

2.4.2 Removal of PCR inhibitors from DNA samples

Whenever a brownish or greyish coloration was observed in DNA extracted from enrichment cultures, possible PCR inhibitors such as polyphenolics, humic and fulvic acids, tannins and melanin, were removed with the OneStep PCR Inhibitor Removal Kit (Zymo Research Europe GmbH, Freiburg, Germany) according to the manufacturer's instructions. The concentration of purified DNA was measured as described in 2.4.1.

2.4.3 Isolation of RNA

Samples for RNA isolation (10 ml) were taken via a syringe and filled into sterile 15 ml falcon tubes. Cells were subsequently harvested by centrifugation (6,000 ×g, 10 min, 10°C) and the supernatant was decanted. The isolation of RNA from the cell pellet was done with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Cells were disrupted via sonication (20% amplitude, 2 seconds on, 2 seconds off; 10 times). After the isolation, co-eluted DNA was digested. Per 50 µl of eluted RNA, 1 µl DNase I (10 U/µl; Roche Diagnostics Deutschland GmbH, Mannheim, Germany), 5 µl tenfold DNase I reaction buffer and 1 µl RiboLock RNase Inhibitor (Thermo Scientific, Schwerte, Germany) were added. The digestion of DNA took place at 37°C for 1 hour. The RNA was then quantified with a Nanodrop 2000 UV-Vis Spectrophotometer. RNA was stored at -80°C until further use.

2.4.4 Polymerase chain reaction (PCR)

All PCR primers used in this study as well as the predicted fragment lengths of PCR products are listed in **Table 2.3**, with the exception of primers used for the detection of the rdhA6 gene, which are listed in the text below.

Endpoint PCR

Endpoint PCR was used for the construction of standards for quantitative PCR or for the detection of inserts in plasmids of transformed *E. coli* XL1 blue. Tenfold PCR buffer, deoxyribonucleotide triphosphates (dNTPs) and Taq polymerase were obtained from Segenetic (Borken, Germany). The twofold master mix for the reaction consisted of the following components: 10× PCR buffer with 15 mM $MgCl_2$ (100 µl), 100 mM dNTPs (2 µl dATP, dTTP, dCTP and dGTP, respectively) and sterile ultra-pure water (392 µl). The reaction mix had a final volume of 25 µl and contained, per reaction, the following components:

12.5 µl	Twofold master mix
5.0 µl	Sterile ultra-pure water
2.5 µl	Forward primer (10 µmol/l)
2.5 µl	Reverse Primer (10 µmol/l)
0.5 µl	Taq polymerase (5 U/µl)
2.0 µl	Genomic DNA (4 ng/µl)

All reactions took place in a MasterCycler Personal thermal cycler (Eppendorf AG, Hamburg, Germany).

For amplification of the full 16S rRNA gene (primer pair 8F/U1517R), the following program was used:

95°C	2 min	initial denaturation	
95°C	1 min	denaturation	30 cycles
55°C	45 sec	primer annealing	
72°C	2 min	extension	
72°C	10 min	final extension	

For amplification of a formyltetrahydrofolate synthetase (FTHFS) fragment specific for *D. hafniense* DCB-2 (primer pair Dhaf0149_-126F/1382R), the following program was used:

95°C	2 min	initial denaturation	
95°C	1 min	denaturation	30 cycles
54°C	45 sec	primer annealing	
72°C	2 min	extension	
72°C	10 min	final extension	

For amplification of a FTHFS fragment designed for acetogens (FTHFS-f/r primer pair), the protocol of Leaphart & Lovell (2001) was used with gDNA of *D. hafniense* DCB-2 as template:

94°C	2 min	initial denaturation	
94°C	30 sec	denaturation	9 touchdown cylces:
63°C	30 sec	primer annealing	annealing temperature is reduced by 1°C
72°C	30 sec	extension	per cycle until 55°C are reached
94°C	30 sec	denaturation	25 cycles
55°C	30 sec	primer annealing	
72°C	30 sec	extension	
72°C	2 min	final extension	

For detection of inserts in the pPrime cloning vector, the following program was used:

95°C	4 min	initial denaturation	
94°C	30 sec	denaturation	25 cycles
58°C	45 sec	primer annealing	
72°C	30 sec	extension	
72°C	10 min	final extension	

For the detection of the rdhA6 gene of *D. hafniense* DCB-2 in enrichment cultures, the primers designed by Mac Nelly et al. (2014) (rdhA6_fw: 5'-GGT AAA ATA CGC TCC AAA CTT C-3'; rdhA6_rv: 5'-TCC GCT TCA GAT GTC ATT TT-3') were used along with the following program:

95°C	4 min	initial denaturation	
94°C	15 sec	denaturation	40 cycles
55°C	30 sec	primer annealing	
72°C	30 sec	extension	
72°C	10 min	final extension	

Quantitative PCR (qPCR)

Quantitative PCR was used to enumerate 16S rRNA and FTHFS gene copies in bacterial enrichment cultures. The signal was generated by the binding of SYBR green to double-stranded DNA. For this, the Maxima SYBR green qPCR Master Mix (Thermo Scientific, Schwerte, Germany) was used. The qPCR standards were obtained from an endpoint PCR amplification of the corresponding target gene (see above). Since the endpoint PCR fragments were not ligated into plasmids, they had to be longer than the corresponding qPCR target sequence in order to ensure an amplification during qPCR even though the 5' and 3' endings of the endpoint PCR fragments might be degraded due to freezing and thawing. After purification of the endpoint PCR fragments from agarose gels (see 2.4.6), their concentration was measured on a Nanodrop 2000 UV-Vis Spectrophotometer. The qPCR standards consisted of dilution series of 10^8 to 10^2 DNA copies/µl of such fragments. For calculation of the DNA copies/µl, the following equations were used:

(1) Molecular weight (MW):

$$MW\ (g\ mol^{-1}) = Size\ of\ fragment\ (bp) \times 660\ g\ mol^{-1}\ bp^{-1}$$

(2) Copy number (CN):

$$CN\ (g\ DNA\ molecule^{-1}) = \frac{MW}{6.022 \times 10^{23}\ mol^{-1}}$$

(3) Copies/µl:

$$DNA\ copies\ (\mu l^{-1}) = \frac{DNA\ concentration\ (g\ \mu l^{-1})}{CN}$$

The qPCR assays were carried out in technical triplicates for each sample of gDNA. All assays stayed in accordance with the MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments), which dictate a reaction efficiency of 90–110% and a calibration curve with $r^2 \geq 0.98$ (Bustin et al., 2009). Typical standard curves including the primer efficiency of each primer pair can be found in the APPENDIX section (**Figure 6.1**).

The reaction mix had a final volume of 10 µl and contained, per reaction, the following components:

5.0 µl	Twofold Maxima SYBR green qPCR Master Mix
3.2 µl	Sterile water, nuclease-free
0.4 µl	Forward primer (10 µmol/l)
0.4 µl	Reverse primer (10 µmol/l)
1.0 µl	Genomic DNA (1 ng/µl)

All reactions were cycled in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Munich, Germany). The protocol used for amplification was the same in all cases except for total FTHFS quantification (see further below):

95°C	10 min	initial denaturation	
95°C	15 sec	denaturation	} 40 cycles
60°C	30 sec	primer annealing	
72°C	30 sec	extension	
Heat from 65–95°C to record melt curves			

The increase in the fluorescence was recorded after each extension step.

For the amplification of total FTHFS, the protocol of Xu et al. (2009) was followed:

94°C	4 min	initial denaturation	
94°C	45 sec	denaturation	9 touchdown cylces:
63°C	45 sec	primer annealing	annealing temperature is reduced by 1°C
72°C	1 min	extension	per cycle until 55°C are reached
94°C	45 sec	denaturation	
55°C	45 sec	primer annealing	30 cycles
72°C	1 min	extension	
Heat from 65–95°C to record melt curves			

The increase in the fluorescence was recorded after each extension step. After the amplification program had finished, qPCR products were separated on a 2% agarose gel to verify the correct length of amplicons as well as the absence of unspecific products. The amount of *Desulfitobacterium* spp.-specific 16S rRNA or FTHFS gene copies was expressed in percent in relation to the total 16S rRNA or FTHFS gene copy number.

Reverse transcription PCR (RT-PCR)

Reverse transcription PCR was used to study the expression of the two FTHFS gene copies in *D. hafniense* DCB-2 (locus tags Dhaf_0149 and Dhaf_0555) at different time points of growth and under various growth conditions. The reverse transcription of extracted RNA and subsequent amplification of cDNA was done with the QIAGEN OneStep RT-PCR Kit (QIAGEN GmbH, Hilden, Germany). The expression levels of Dhaf_0149 and Dhaf_0555 were compared to the one of RNA polymerase subunit beta (rpoB), a house-keeping gene. Furthermore, a sample which contained 2 µl of genomic DNA (50 ng/µl) of *D. hafniense* DCB-2 instead of RNA was used as a positive control. The reaction mix had a final volume of 25 µl and contained, per reaction, the following components:

5.0 µl	Fivefold RT-PCR-buffer
2.5 µl	Forward primer (10 µmol/l)
2.5 µl	Reverse primer (10 µmol/l)
1.0 µl	dNTPs (10 mmol/l)
1.0 µl	Enzyme mix (reverse transcriptase and Taq polymerase)
13 µl	1 µg RNA and RNase-free water

Each reaction mixture was halved by pipetting 12.5 µl into a separate Eppendorf tube. One of both tubes was designated as minus RT (-RT) control and was frozen at -20°C along with the positive control tubes until the reverse transcription step had ended. The other tube was designated as plus RT (+RT) tube. The reverse transcription and subsequent amplification of cDNA were both performed in a MasterCycler Personal thermal cycler (Eppendorf AG, Hamburg, Germany). For reverse transcription, all +RT tubes were incubated at 50°C for 60 min. Afterwards, -RT and positive control tubes were retrieved from the freezer and amplified along with the +RT tubes. For amplification of cDNA, the program used was as follows:

95°C	15 min	initial denaturation	
95°C	1 min	denaturation	25 cycles
60°C	30 sec	primer annealing	
72°C	30 sec	extension	
72°C	10 min	final extension	

All RT-PCR products were separated on a 2% agarose gel and documentation was carried out as described in 2.4.5.

Table 2.3: List of primers used in this study. The expected sizes of PCR products were derived from primer alignments with the genome sequence of *D. hafniense* DCB-2. The expected product size for acetogenic FTHFS was taken from Xu et al. (2009). Abbreviations: *Dsf* (*Desulfitobacterium* spp.), FTHFS (formyltetrahydrofolate synthetase), MCS (multiple cloning site), rpoB (RNA polymerase subunit B).

Primer pair	Sequence (5' → 3')	Target	Product length (base pairs)	PCR type	Reference
8F U1517R	AGA GTT TGA TCC TGG CTC AG ACG GCT ACC TTG TTA CGA CTT	16S rRNA (Bacteria)	1529	Endpoint PCR	Weisburg et al. (1991)
341F 543R	CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	16S rRNA (Bacteria)	192	qPCR	Muyzer et al. (1993)
356F De2_YR	ACT CCT ACG GRA GGC WGC CYA RGT TTT CAC ACC AGA CTT	16S rRNA (*Dsf*)	279	qPCR	García et al. (2012) Mac Nelly (2015)
FTHFS-f FTHFS-r	TTY ACW GGH GAY TTC CAT GC GTA TTG DGT YTT RGC CAT ACA	FTHFS (total)	1098	Endpoint PCR	Leaphart & Lovell (2001)
fhs1 FTHFS-r	GTW TGG GCW AAR GGY GGM GAA GG GTA TTG DGT YTT RGC CAT ACA	FTHFS (total)	252 250[a]	qPCR	Xu et al. (2009) Leaphart & Lovell (2001)
Dhaf0149_-126F Dhaf0149_1382R	TGT TAA TCC AAG CAG GGA ATC T ACA CCA TCG GCA CCA TAA AC	FTHFS (*Dsf*)	1508	Endpoint PCR	This study
Dhaf0149_127F Dhaf0149_409R	AGT GCT TGG GAA CGC CTY AAR GAG TCG AAG TAA TRG CRT GGA	FTHFS (*Dsf*)[b]	283	qPCR, RT-PCR	This study
Dhaf0555_1405F Dhaf0555_1665R	AGC RCC AAG GAT ATC GAG RRY GGA AAT CYT RCC YGT GTT GTC	FTHFS (*Dsf*)[c]	261	RT-PCR	This study
rpoB-f rpoB-r	GAT TCG GGC TTT GGG TTA TGC CGC AGA CGC TTG TAG ATT TCC	rpoB	138	RT-PCR	Reinhold et al. (2012)
T7-f SP6-r	GTA ATA CGA CTC ACT ATA G CAT TTA GGT GAC ACT ATA G	pPrime cloning vector (MCS)	Insert-dependent	Endpoint PCR	Perfect PCR Cloning Kit manual

[a] Average product length for acetogenic bacteria according to Xu et al. (2009)
[b] Primer pair targets locus tag Dhaf_0149 and homologs
[c] Primer pair targets locus tag Dhaf_0555 and homologs

2.4.5 Agarose gel electrophoresis

PCR products were separated on agarose gels to verify the corresponding length of the fragments and/or the presence of unspecific products. The gels consisted of 0.8–2% (w/v) agarose, TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and ethidium bromide (0.5 µg/ml). PCR products were mixed with 5× loading buffer. Approximately 5 µl were then pipetted into the gel's lanes. HyperLadder I, II and IV (Bioline, Luckenwalde, Germany) were used as references for the fragments' lengths. The separation of DNA took place in a Bio-Rad GT Agarose Gel Electrophoresis Sub-Cell with a PowerPac HC Power Supply system (Bio-Rad Laboratories GmbH, Munich, Germany) with a voltage of 90 V, followed by the documentation of the gels with a Gel iX20 Imager device (Intas Science Imaging Instruments GmbH, Göttingen, Germany).

2.4.6 Excision and purification of DNA fragments from agarose gels

DNA bands were excised under UV light with a scalpel and transferred to sterile Eppendorf tubes. The extraction of separated DNA from the agarose gel was carried out with the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Eluted DNA was separated on a 0.8% agarose gel to verify the successful excision and purification.

2.4.7 Ligation of DNA fragments to pPrime Cloning Vector

DNA fragments were ligated into the pPrime Cloning Vector (for a vector map, see **Figure 2.2**) using the Perfect PCR Cloning Kit (5PRIME GmbH, Darmstadt, Germany) according to the manufacturer's instructions. A total of 4 µl of purified qPCR product were used for the ligation, which lasted between 30 min–2 h at a temperature of 4°C. Afterwards, an additional incubation step at 70°C for 10 min was used to inactivate the ligase. The resulting vector construct was used for transformation of competent *E. coli* XL1 blue cells without further delay.

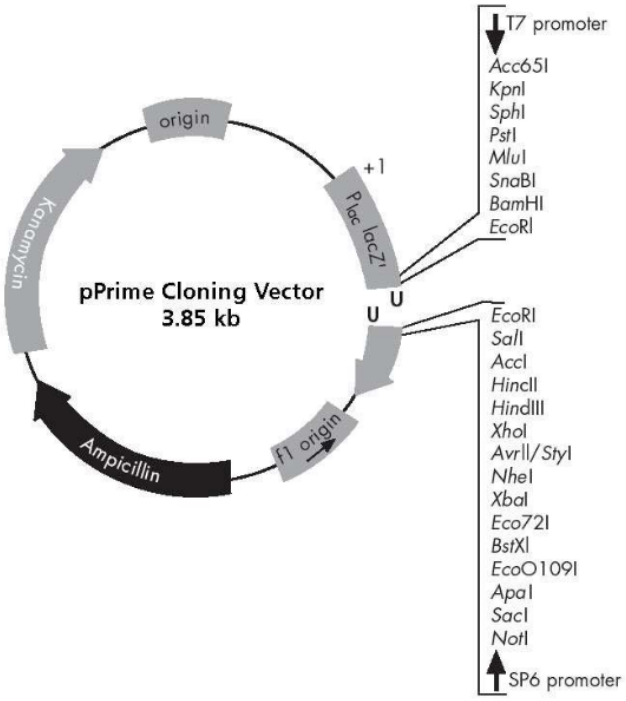


Figure 2.2: pPrime Cloning Vector map. The U overhangs of the linearized pPrime Cloning Vector are shown, as well as the restriction sites of the multiple cloning site. The vector map was obtained from the Perfect PCR Cloning Kit manual.

2.4.8 Transformation of competent *E. coli* XL1 blue cells

The transformation of *E. coli* XL1 blue was based on a heat shock procedure. Competent *E. coli* XL1 blue cells were thawed on ice for 30 min. The cells were then added directly to the ligation mix containing the vector construct and incubated for additional 30 min on ice. The heat shock was then triggered by incubating the cells for 30–45 sec at 42°C, followed by a short incubation (1 min) on ice. Next, 750 µl LB medium were added and cells were incubated for 1 h at 37°C and 1000 rpm. The cells were subsequently plated on solid LB media, containing 100 µg/ml ampicillin for antibiotic selection, and were incubated upside down over night at 37°C. Circular colonies could be observed on the plates the next morning. The presence of the insert within the multiple cloning site (MCS) of the plasmid was verified via colony PCR. For this, clones were picked with sterile toothpicks under a clean bench and suspended in 20 µl sterile ultra-pure water. To conserve the clone in case of a positive PCR amplification, 2 µl of this suspension were pipetted on a petri dish containing solid LB medium (100 µg/ml ampicillin) and incubated for 24 h at 37°C. For the PCR, 2 µl of the cell suspension were added as template to the PCR reaction mix and the amplification was performed as described in 2.4.4 (*Endpoint PCR*). The PCR products were then run on a 0.8% agarose gel to verify the presence of the insert. For plasmid preparation of positive tested colonies (see 2.4.9), the newly grown clones

were transferred to 5 ml of liquid LB medium containing 100 µg/ml ampicillin. The clones were allowed to grow for 24 h at 28°C.

2.4.9 Plasmid preparation and Sanger sequencing

Plasmid preparation was performed using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Schwerte, Germany). The method is based on the alkaline lysis of cells. For this, 1.5 ml of grown *E. coli* XL1 blue cultures were centrifuged for 1 min at 16,000 ×g. The supernatant was removed and the cell pellet was used for the extraction of plasmids. After the plasmids had been eluted following the kit procedure, they were separated on a 0.8% agarose gel to verify the integrity of the plasmid. If this was the case, a mixture of 8 µl plasmid solution and 2 µl T7 primer solution (10 µmol/l) were sent to GATC Biotech (Cologne, Germany) for Sanger sequencing. The resulting sequences were aligned using the BLAST N tool (Altschul et al., 1990).

2.4.10 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization was used for the visual detection of *Desulfitobacterium* spp. in enrichment cultures. The protocol established by Yang et al. (2005) was followed. The Cy3-labelled probes used were taken from the same study. Their sequences were as follows: Dsf440, 5'-Cy3-TAC CGT TCG TCC CTG AAG-3'; Dsf475, 5'-Cy3-CTC AGG TAC CGT CAT GTA AG-3'. The specificity of the probes was tested in mixed cultures consisting of *Desulfitobacterium* spp. and *Acetobacterium dehalogenans* in a 1:1 ratio. For each FISH experiment, a negative control and a positive control were performed with *D. hafniense* DCB-2 cells. The negative control lacked both probes used for hybridization, which were substituted by sterile ultra-pure water. No signals that might indicate background noise were observed in negative controls. The fixation of cells was performed on the day prior to the hybridization with 4% (w/v) paraformaldehyde (PFA) solved in phosphate buffered saline (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.2). A 2 ml sample of each enrichment culture was taken and centrifuged for 1 min at 16,000 ×g. The supernatant was removed and the cells were washed and resuspended in 750 µl PBS. Then, 250 µl of PFA solution were added. The fixation took place for 16 h at a temperature of 4°C. The cells were then centrifuged for 1 min at 16,000 ×g and the supernatant was removed. Cells were subsequently washed in PBS and resuspended in the same buffer at a desired volume. Then, 10 µl of fixed cells were pipetted to gelatin-coated wells of a microscope well-slide and allowed for the buffer to evaporate and the cells to adhere to the gelatin surface for approximately 2–3 hours. Afterwards, cells were dehydrated in a series

of ethanol solutions (50%, 80% and 96% [v/v]; 3 min at room temperature in each solution). The slides were left to dry and 10 µl of lysozyme (400 U/µl) were pipetted to each well. Slides were then incubated for 5 min at 4°C. The lysozyme was removed by washing the slides in ultra-pure water. The hybridization was started by the addition of 2.5 µl of the probe mixture to each well in a humid enclosed atmosphere. This mixture contained 15 ng of each probe (Dsf 440 and Dsf475) in 900 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01% (w/v) SDS and 5% (v/v) formamide (AppliChem GmbH, Darmstadt, Germany). The hybridization was performed at 42°C for 2 h. Afterwards, cells were washed for 15 min in a washing buffer (636 mM NaCl, 20 mM Tris-HCl, 10 mM EDTA, 0.01% [w/v] SDS) at 48°C. The slides were then rinsed in ultra-pure water and left to dry. The DNA of the bacteria was then stained by addition of 10 µl of 4'-6-diamidino-2-phenylindole (DAPI; 1 ng/µl) and incubating for 3 min at room temperature in the dark. DAPI was washed off by rinsing the slides in ultra-pure water. After the slides had dried, 10 µl of *SlowFade* antifade reagent (Life Technologies GmbH, Darmstadt, Germany) were pipetted to each well and the slide was covered with a cover slip. Epifluorescence images were taken with an AxioCam MRm equipped to an Axio Scope A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.4.11 Illumina MiSeq sequencing of 16S rRNA genes

The Illumina MiSeq sequencing of 16S rRNA genes for analysis of microbial communities from soils and enrichment cultures was carried out at the Research and Testing Laboratory (Lubbock, Texas, United States of America) using 2 × 300 bp runs. The following samples were sequenced: genomic DNA originating from all soils, genomic DNA originating from the sub-cultivation of each enrichment that contained the maximum amount of *Desulfitobacterium* spp. 16S rRNA gene copies and genomic DNA originating from the last sub-cultivation of each enrichment. In case of enrichment cultures amended with syringate/thiosulfate, for which biological duplicates existed, only one replicate was chosen for enrichment. All samples were diluted to a concentration of 20 ng/µl, if possible.

The following workflow, the description of which was obtained from the official Illumina webpage (http://www.illumina.com), was performed at the Research and Testing Laboratory in Lubbock (USA). It starts with the random fragmentation, also called tagmentation, of genomic DNA via transposomes, which tag both ends of the DNA with adapter sequences. One of these adapter sequences contains the sequencing primer binding site for the first application sequencing read (forward read). Then, the 16S rRNA gene is amplified in a reduced cycle amplification using the primers 28F (5'-GAG TTT GAT CNT GGC TCA G-3') and 519R (5'-

GTN TTA CNG CGG CKG CTG-3'), which target the hypervariable regions 1–3 (V1–V3) of the 16S rRNA gene. One primer contains an attachment site for the flow cell, while the second contains the sequencing primer binding sites for the index read and for the second application read (reverse read). A third primer in the PCR adds the index as well as a second flow cell attachment site to the PCR product. Both amplification steps are summarized in **Figure 2.3**.

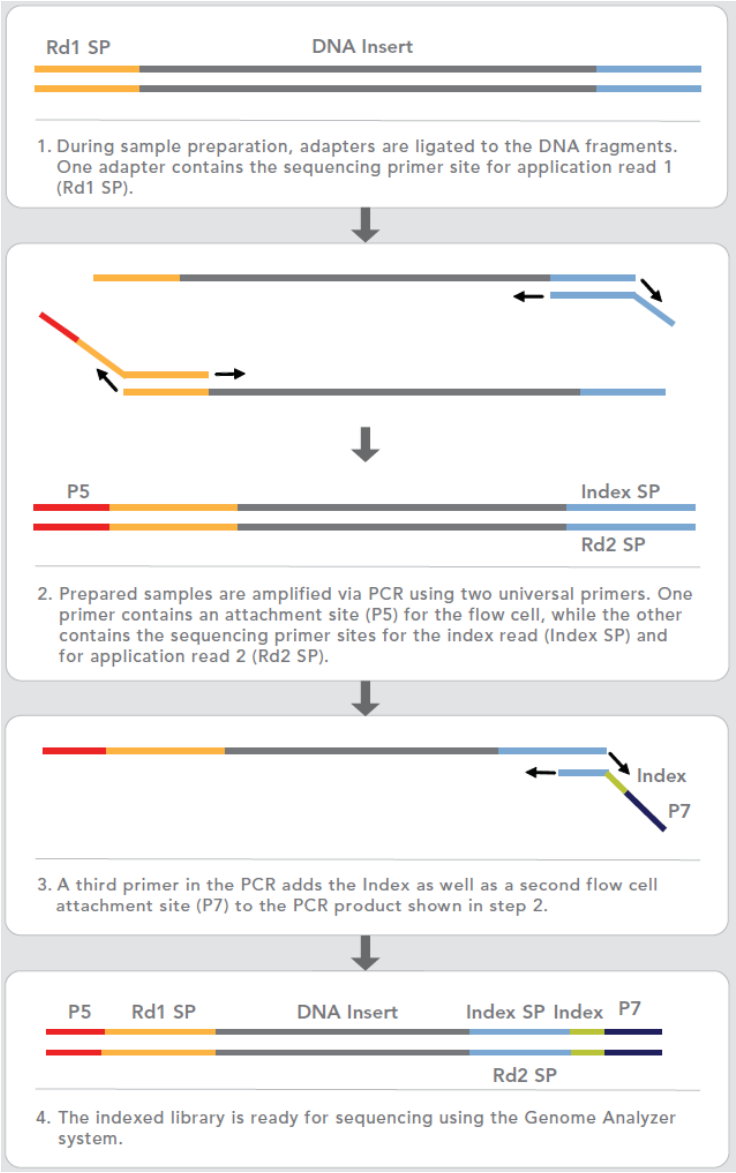


Figure 2.3: Amplicon design during Illumina sample preparation. Image © Illumina (http://www.illumina.com).

The library is then applied and tethered to a flow cell. The double-stranded DNA is denatured after applying a sodium hydroxide solution and the single strands are randomly bound to complimentary adapter sequences across the flow cell surface. Through the addition of unlabeled dNTPs a solid-phase bridge amplification is initiated by the bending of a fully amplified strand to bind to a closely located flow cell surface sequence. This results in the

formation of clusters of double-stranded DNA bridges. After the amplification ends, the reverse reads are cleaved and washed off, leaving only the forward strands on the flow cell. The 3'–OH ends are blocked to prevent unwanted priming.

To initiate the sequencing, the first sequencing primer, fluorophore-labeled dNTPs (reversible terminators) and DNA polymerase are applied to the flow cell. The dNTPs are blocked at their 3'–OH end to prevent the incorporation of more than one nucleotide into the growing strand per cycle. For this study, 2 × 300 bp runs were performed. This means that two sequencing runs were done per sample, each consisting of 300 cycles in which only one nucleotide is incorporated into the amplifying DNA strand at a time.

Sample multiplexing (the assembly of reads belonging to one DNA strand) involves a total of three sequencing reads (see **Figure 2.4**). The first read corresponds to the forward read ("application read 1"). After the incorporation of the first nucleotide, lasers excite the fluorescent tags at the 3'–OH end of each cluster and images are captured via a camera. The identity of the first base is recorded. In subsequent cycles, the process of adding sequencing reagents, removing unincorporated bases and capturing the signal of the next base to identify is repeated. As each base is immediately recorded after its incorporation to the growing DNA strand, this process is called "sequencing by synthesis".

After the forward read has been completed, its product is washed away and the index 1 sequencing primer is annealed to the same strand to produce a short base pair index read. For the reverse read, the 3'–OH end of the original template strand is deprotected. The template then folds over and binds to a closely located adapter sequence on the flow cell surface. Then, the index 2 read is produced and washed off. Subsequently, a polymerase extends the second flow cell oligonucleotide to produce the reverse complementary strand of the original template strand. The strands are again denatured and their 3'–OH ends again blocked to avoid unwanted priming. The original forward strand is cleaved off and washed away to retain solely the complementary strand on the flow cell. The reverse read ("application read 2") then begins with the introduction of the second sequencing primer. Upon its completion, the read product is washed away. Since each strand was read in the forward and reverse direction, this process is also called "paired end sequencing".

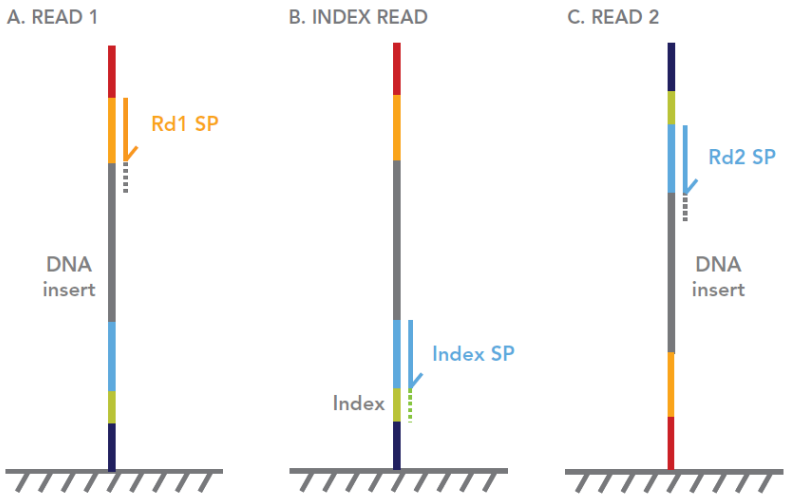


Figure 2.4: Sequencing procedure. First, the forward read ("read 1") is performed (A), followed by the index read (B). After synthesis of the reverse complementary strand, a second index read and the reverse read ("read 2") are performed (C). Abbreaviations: Rd1 SP (read 1 sequencing primer), Rd2 SP (read 2 sequencing primer). Image © Illumina (http://www.illumina.com).

The reads obtained are separated according to the corresponding indices introduced during sample preparation. For each sample, reads with similar stretches of base calls are locally clustered, forward and reverse reads are paired and then compared to a reference genome. The data is then run through a pipeline for further analysis (see 2.5.3).

2.5 Bioinformatic methods

2.5.1 Mapping of putative demethylase gene clusters in *Desulfitobacterium* spp.

Mapping of putative demethylase systems was carried out with the NCBI BLAST Server (Altschul et al., 1990) and the Integrated Microbial Genomes (IMG) System (Markowitz et al., 2014). The templates used for BLAST and IMG searches were the amino acid sequences of the three components (methyltransferase I, MT I; corrinoid protein, CP; methyltransferase II, MT II) of an *O*-demethylase previously characterized in *D. hafniense* DCB-2 (Studenik et al., 2012). The corresponding locus tags were Dhaf_4610 (MT I; GenBank accession no. ACL22611.1), Dhaf_4611 (CP; GenBank accession no. ACL22612.1) and Dhaf_4612 (MT II; GenBank accession no. ACL22613.1). In *D. hafniense* DCB-2, MT I-encoding genes (length ≈ 1000 bp) are annotated as uroporphyrinogen-III-decarboxylases, which have a molecular mass of about 37 KDa; CP-encoding genes (length ≈ 600 bp) are annotated as cobalamin-binding proteins, which have a molecular mass of about 21 KDa; and MT II-encoding genes (length ≈ 800 bp) are typically annotated as pterin-binding enzymes, which have a molecular mass of about 29 KDa. For the identification of COG3894 proteins that might function as AE, the amino acid

sequence of Dhaf_2573 (AE; GenBank accession no. ACL20600.1) was used in BLAST and IMG searches. COG3894-encoding genes can vary in length and are often annotated as metal-binding enzymes or ferredoxins. Putative demethylases were mapped in the genomes of *D. dehalogenans*, *D. dichloroeliminans*, *D. metallireducens* and *D. hafniense* strains DP7, PCE-S, PCP-1, TCE1, TCP-A and Y51 (Y51: Nonaka et al., 2006). However, a discrimination between *Cl*-, *N*-, *O*- and *S*-demethylases was not possible. All identified demethylase operons in *Desulfitobacterium* spp., defined as gene clusters containing at least two of the catalytic components of the *O*-demethylation reaction (MT I, CP, MT II), were compared in terms of organization and similarity of the encoded proteins.

2.5.2 FTHFS-primer design

Specific primers targeting the two FTHFS gene copies in *D. hafniense* DCB-2 (locus tags Dhaf_0149 and Dhaf_0555) and their homologs in the genomes of other *Desulfitobacterium* spp. (see **Table 3.9**) were designed in this study. For this, the amino acid sequences of the corresponding enzymes were obtained from the GenBank (Benson et al., 2013) or IMG (Markowitz et al., 2014) databases and aligned with Clustal Omega v. 1.2.1 (Goujon et al., 2010) along with FTHFS amino acid sequences from acetogenic (*Acetobacterium woodii*, accession no. WP_014355320.1; *Clostridium aceticum*, accession no. WP_044823346.1; *Eubacterium cellulosolvens*, accession no. EIM56292.1; *Moorella thermoacetica*, accession no. WP_011391657.1; *Sporomusa ovata*, accession no. WP_021171366.1) and non-acetogenic (*Bacillus azotoformans*, accession no. WP_003331389.1; *Desulfosporosinus orientis*, accession no. AET65832.1) bacteria. Amino acid patterns that distinguished the FTHFS copies of *Desulfitobacterium* spp. from other bacteria (see APPENDIX section, **Figure 6.2** and **Figure 6.3**) were used for the design of specific primers (see **Figure 2.5**). The melting temperature of the primer pairs as well as the potential for formation of hairpin structures, homo-dimers and hetero-dimers was determined with the OligoAnalyzer 3.1 tool (Owczarzy et al., 2008).

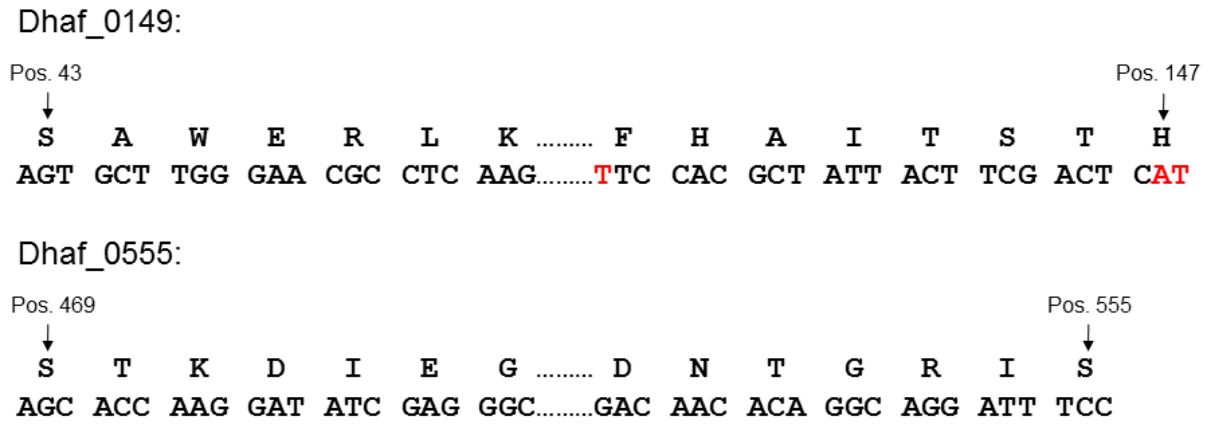


Figure 2.5: Amino acid residues (upper row) and corresponding codons (lower row) that were chosen for the design of primers targetting both FTHFS gene copies in *Desulfitobacterium* spp., shown for *D. hafniense* DCB-2 (5' to 3' orientation). The indicated positions belong to the amino acid residues. Nucleotides labelled in red were excluded from the Dhaf_0149 reverse primer.

The specificity of both primer pairs was tested with genomic DNA of *Desulfitobacterium* spp., *Acetobacterium dehalogenans* and *Sporomusa ovata* in PCR experiments as well as by generation of clone libraries from enrichment cultures. No unspecific products were detected with any of both primer pairs. Furthermore, both primer pairs were designed in a way that the non-targeted FTHFS copy in the genome of *Desulfitobacterium* spp. is discriminated during amplification. This study therefore presents two genus- and gene-specific primer pairs (see **Table 2.3**).

2.5.3 Processing of Illumina MiSeq data

The Research and Testing Laboratory (Lubbock, Texas, USA) provided two .fastq files for every sample: the first one containing the forward reads, the second one containing the reverse reads (paired ends). Both reads were joined and processed using mothur (Schloss et al., 2009) according to the standard operating procedure (Kozich et al., 2013). The complete MiSeq SOP is available online at http://www.mothur.org/wiki/MiSeq_SOP (last accessed: November 2014). The used script is presented in the APPENDIX section (**Script 6.1**). **Figure 2.6** depicts the workflow of the pipeline process.

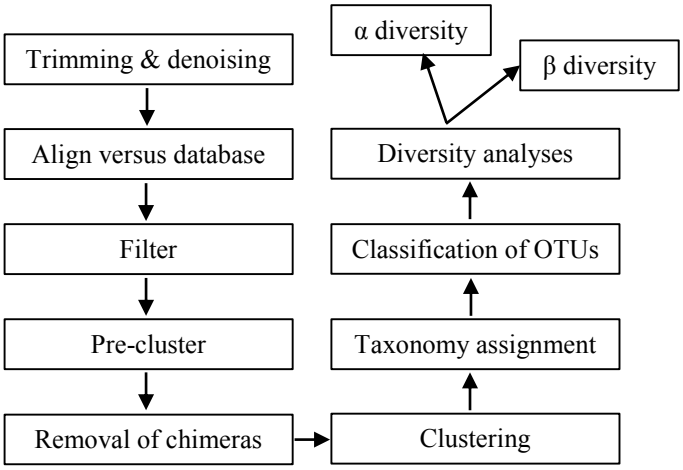


Figure 2.6: Typical workflow for processing Illumina MiSeq data. The workflow starts with the "trimming & denoising" step and follows in the direction of the arrows.

The processing consisted of trimming steps to remove sequences excluded of a chosen length interval (in this study, all sequences >560 and <430 bp were excluded), sequences with ambiguous bases and sequences with homopolymers (in this case all sequences with >8 homopolymers). In this step, the dataset was also denoised and all identical sequences were merged into a unique sequence to reduce the data load. Afterwards, the remaining sequences were aligned against the SILVA database (Quast et al., 2013) and then pre-clustered. Chimeras were identified and removed with UCHIME (Edgar et al., 2011; Schloss et al., 2011). Finally, the remaining sequences were classified with the naïve Bayesian Classifier (Wang et al., 2007) with a minimum bootstrap support of 80%, followed by a phylotype analysis and assignment of operational taxonomic units (OTUs). In this study, an OTU is defined as a unique sequence that can occur multiple times within a sample, and that shares at least a 97% sequence identity with known 16S rRNA gene sequences. In this study, the number of OTUs within a sample does not depict the absolute number of reads that can be assigned to a taxon, but the diversity of sequences that were identified for said taxon.

Afterwards, studies on the α and β diversity of samples were conducted, also with mothur. For this, sub-samples consisting of 4538 randomly picked sequences from each sample were used to assess different diversity parameters: rarefaction, Simpson's reciprocal index (Simpson, 1949), Jaccard's dissimilarity coefficient (Jaccard, 1901) and Yue & Clayton's dissimilarity coefficient (Yue & Clayton, 2005). The number 4538 corresponded to the lowest amount of reads found in one sample, namely in the second sub-cultivation step of the gleysol enrichment culture (syringate/thiosulfate couple).

Simpson's diversity index (D) describes the probability of picking the same OTU twice if two random OTUs are picked from a sample. If no diversity exists in a sample (e.g. only one species is present), D will equal 100% (or 1). The ideal diversity of 0% describes the hypothetical case of infinite diversity, in which there is no chance of picking the same species randomly twice because each species is represented by only one individual. Simpson's reciprocal index (D') starts with 1 as the lowest value (in this case, D = 100% or 1). The higher D', the higher the diversity in a sample. The maximum D' value equals the number of species in the sample. Simpson's diversity index (D) and Simpson's reciprocal index (D') were calculated according to the following formulas by mothur:

(1) Simpson's diversity index $$D = \sum_{i=1}^{S} \left[\frac{n_i(n_i-1)}{N(N-1)}\right]$$

(2) Simpson's reciprocal index $$D' = {}^{1}/_{D}$$

Where S equals the number of observed OTUs, n_i equals the number of individuals in the *i*th OTU and N equals the total number of individuals in the community.

Jaccard's as well as Yue & Clayton's indices are indicators for the dissimilarity between the communities of two samples based on species richness, evenness and abundance. While Jaccard's index focusses on the similarity in community membership, the Yue & Clayton index focusses on the similarity in community structure. Mothur calculates both coefficients according to the following formulas:

(3) Jaccard's measure of dissimilarity $$D_J = \frac{S_{AB}}{S_A + S_B - S_{AB}}$$

Where S_{AB} equals the number of shared OTUs between communities A and B, S_A equals the number of OTUs in community A and S_B equals the number of OTUs in community B.

(4) Yue & Clayton's measure of dissimilarity $$D\theta_{YC} = 1 - \frac{\sum_{i=1}^{S_T} A_i B_i}{\sum_{i=1}^{S_T}(A_i - B_i)^2 + \sum_{i=1}^{S_T} A_i B_i}$$

Where S_T equals the total number of OTUs in communities A and B, A_i equals the relative abundance of OTU *i* in community A and B_i equals the relative abundance of OTU *i* in community B.

3 RESULTS

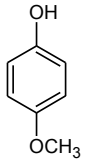
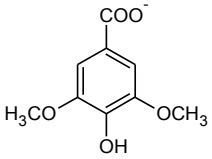
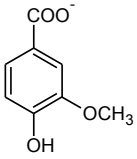
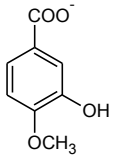
3.1 Growth of *Desulfitobacterium* spp. with phenyl methyl ethers in the presence of different electron acceptors

The utilization of the methyl moiety of methoxylated aromatics as electron donor by *Desulfitobacterium* spp. was first described for *Desulfitobacterium hafniense* strains DCB-2 and PCE-S (Neumann et al., 2004). The phenyl methyl ether vanillate was demethylated to protocatechuate in the presence of the electron acceptor fumarate and, in the case of *D. hafniense* DCB-2, also Cl-OHPA. In contrast to the acetogenic bacteria, no *O*-demethylation occurred in the presence of CO_2 as terminal electron acceptor. Recently, an *O*-demethylase of *D. hafniense* DCB-2 was characterized (Studenik et al., 2012). Additionally, several putative demethylase systems were identified in its genome, indicating the ability of *D. hafniense* DCB-2 to cleave the ether bond of a variety of methoxylated aromatic compounds.

In order to assess and elucidate the possible extent of phenyl methyl ether demethylation in soils by desulfitobacteria, pure cultures of *D. chlororespirans*, *D. dehalogenans*, *D. metallireducens* and various *D. hafniense* strains were tested for *O*-demethylation of four phenyl methyl ethers in combination with different electron acceptors in growth experiments. The phenyl methyl ethers used were 4-hydroxyanisole, syringate, vanillate and isovanillate. Since an electron acceptor different from CO_2 is needed, compounds that are expected to occur in natural environments and that might play a physiological role for this process, such as nitrate, thiosulfate and Fe(III), were tested for their reduction upon desulfitobacterial *O*-demethylation. Fumarate was used as a control.

All species with the exception of *D. metallireducens* were able to grow on all electron acceptors tested in combination with at least three of the supplied phenyl methyl ethers by cleaving the ether bond (**Table 3.1**).

Table 3.1: Maximal rate of phenyl methyl ether consumption by growing *Desulfitobacterium* spp. (µM h^{-1}) in the presence of fumarate (a; first row), nitrate (b; second row) or thiosulfate (c; third row) as electron acceptor. Consumption rates for Fe(III) are not shown. Adapted from Mingo et al. (2014).

Species and strain		**Demethylation rates (µM h^{-1})**			
		4-Hydroxy-anisole	Syringate	Vanillate	Isovanillate
					
Desulfitobacterium hafniense	DCB-2	37[a]	39	25	88
		40[b]	28	25	8
		57[c]	37	34	8
	DP7	19	26	98	61
		20	14	24	16
		35	17	15	19
	G2	<1	75	23	40
		<1	21	21	15
		<1	31	26	23
	PCE-S	<1	54	63	69
		<1	17	21	19
		<1	26	35	43
	PCP-1	83	14	54	63
		35	<1	10	22
		24	<1	8	18
	TCP-A	38	39	23	35
		15	19	23	39
		35	14	7	10
	Y51	<1	25	17	80
		<1	49	30	22
		<1	16	10	30
D. dehalogenans		<1	29	28	90
		<1	14	10	10
		<1	7	10	20
D. chlororespirans		10	67	46	16
		3	25	27	30
		7	26	25	30

Short lag phases of approximately one to two days were observed after inoculation of the growth media with pre-cultures grown on 40 mM pyruvate and 40 mM fumarate. Vanillate and isovanillate were demethylated to protocatechuate (3,4-dihydroxybenzoate), 4-hydroxyanisole was demethylated to hydroquinone (benzene-1,4-diol) and syringate was demethylated via 3-*O*-methylgallate (3,4-dihydroxy-5-methoxybenzoate) to gallate (3,4,5-trihydroxybenzoate). Growth stopped once the phenyl methyl ether (and its intermediate in the case of syringate) had been completely demethylated. No inhibitory effect on growth was observed with the applied substrate concentrations (see 2.2.1). No growth occurred when either phenyl methyl ethers or fumarate, nitrate, thiosulfate or Fe(III)citrate were supplied as sole growth substrates with the exception of *D. hafniense* TCE1, which also grew with fumarate as sole substrate. Since the growth behavior of this strain did not yield unambiguous results, *D. hafniense* TCE1 was excluded from further studies. All *Desulfitobacterium* species and strains tested could not grow in the presence of phenyl methyl ethers as sole energy substrates, neither in carbonated growth medium under a N_2 (100%) atmosphere nor under a CO_2:N_2 (80:20) atmosphere, confirming previous findings that stated the inability of these bacteria to utilize CO_2 as electron acceptor (Neumann et al., 2004; Kreher et al., 2008).

Syringate, vanillate and isovanillate were converted by all *Desulfitobacterium* spp. tested. In contrast to these, the demethylation of 4-hydroxyanisole was restricted to only some of the desulfitobacteria tested. *D. hafniense* strains G2, PCE-S and Y51 as well as *D. dehalogenans* were not able to utilize 4-hydroxyanisole, indicating that they lacked either the ability to cleave the ether bond of this substrate or a membrane transporter needed for uptake. For most species and strains, no preference existed concerning the phenyl methyl ether applied, which is reflected in similar conversion rates of the applied phenyl methyl ethers when using the same electron acceptor (e.g. fumarate, see **Table 3.1**). During cultivation with fumarate as the electron acceptor, the conversion rate was typically higher in comparison with nitrate, thiosulfate or Fe(III). When comparing growth yields, no significant differences could be observed during growth in the presence of fumarate, nitrate, thiosulfate or Fe(III) as electron acceptors. The growth yield was, however, slightly higher in the presence of fumarate and nitrate (see **Figure 3.1** and **Figure 3.2**).

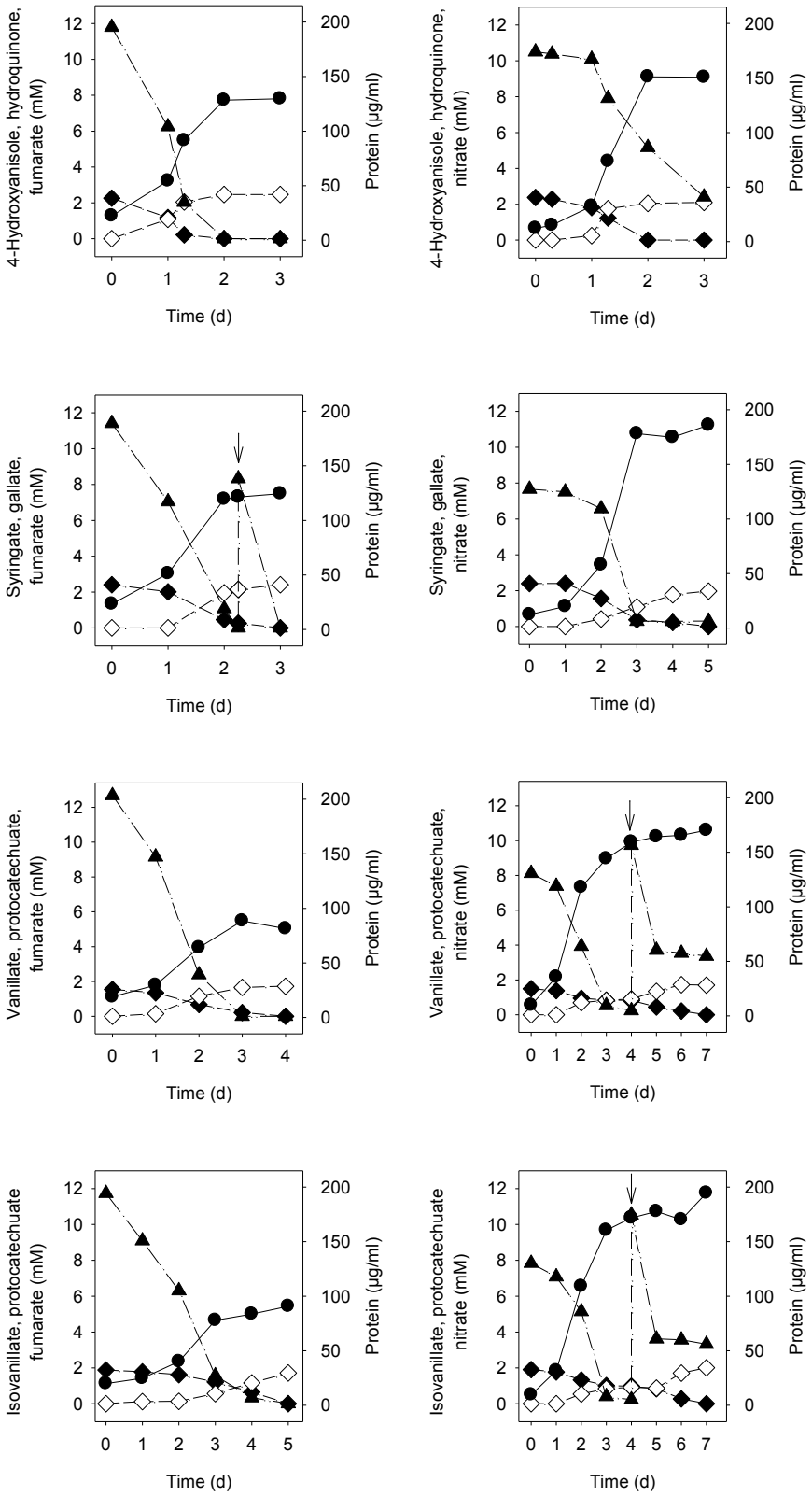


Figure 3.1: Growth of *D. hafniense* DCB-2 on 4-hydroxyanisole (first row), syringate (second row), vanillate (third row) and isovanillate (fourth row) in the presence of fumarate (left column) and nitrate (right column). Figure legend: ♦, phenyl methyl ether; ◊, demethylated product; ▲, electron acceptor; ●, protein concentration. Arrows indicate the replenishment of the electron acceptor.

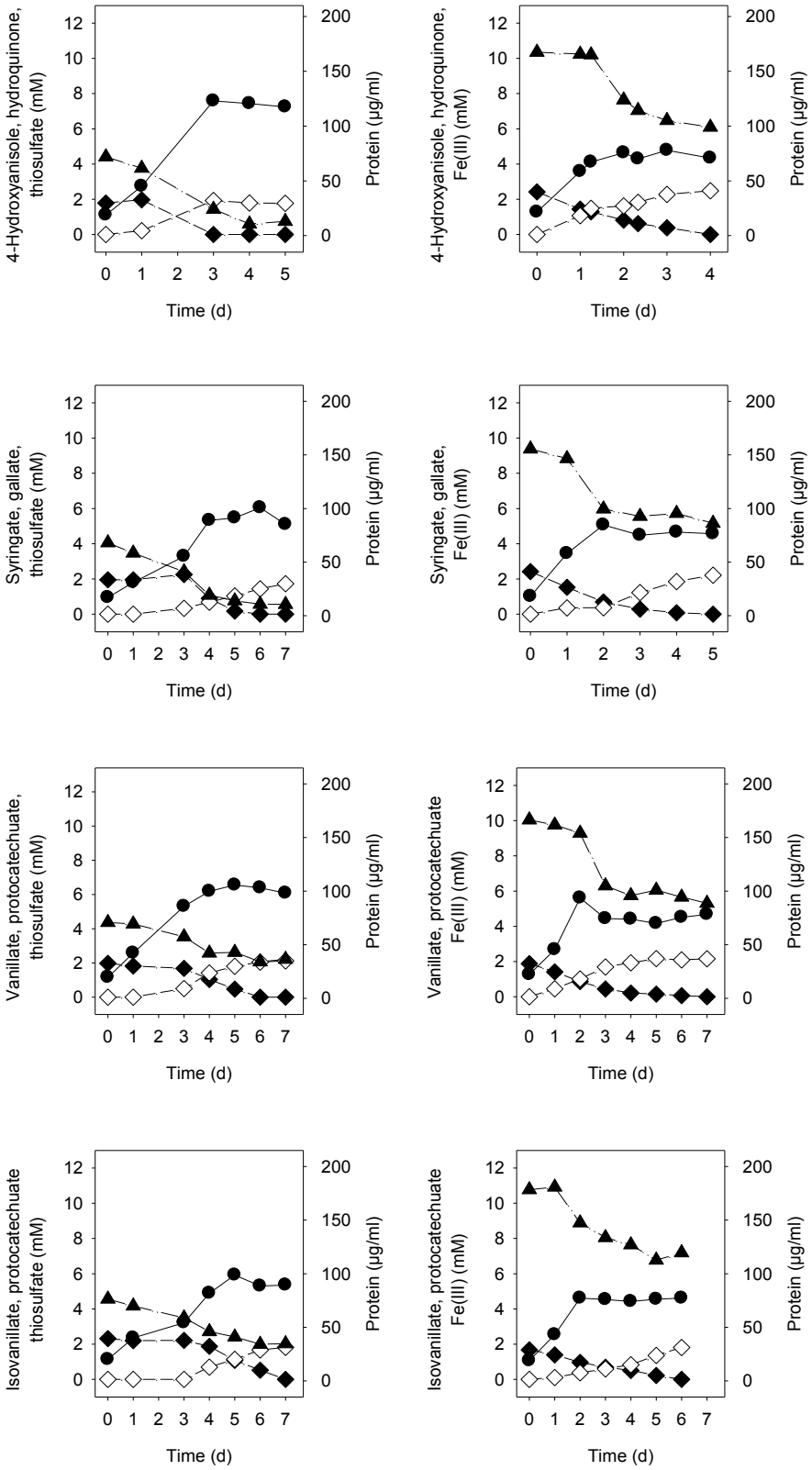


Figure 3.2: Growth of *D. hafniense* DCB-2 on 4-hydroxyanisole (first row), syringate (second row), vanillate (third row) and isovanillate (fourth row) in the presence of thiosulfate (left column) and Fe(III) (right column). Figure legend: ♦, phenyl methyl ether; ◊, demethylated product; ▲, electron acceptor; ●, protein concentration.

The measured stoichiometry of fumarate, nitrate, thiosulfate or Fe(III) reduced per mol methyl group consumed often differed from the values expected according to the following reaction equations:

(1) $R\text{-}O\text{-}CH_3 + 3\ fumarate + 2\ H_2O \rightarrow R\text{-}OH + 3\ succinate + CO_2$

(2) $4\ R\text{-}O\text{-}CH_3 + 3\ NO_3^- + 6\ H^+ \rightarrow 4\ R\text{-}OH + 3\ NH_4^+ + 4\ CO_2 + H_2O$

(3) $4\ R\text{-}O\text{-}CH_3 + 3\ S_2O_3^{2-} + 6\ H^+ \rightarrow 4\ R\text{-}OH + 6\ H_2S + 4\ CO_2 + H_2O$

(4) $R\text{-}OCH_3 + 6\ Fe^{3+} + 2\ H_2O \rightarrow R\text{-}OH + CO_2 + 6\ Fe^{2+} + 6\ H^+$

In the case of fumarate, nitrate and thiosulfate, a higher consumption than the expected one could be observed. This finding can be explained for fumarate by the disproportionation of this substrate, which was observed in growth media with fumarate as the sole carbon source. However, no *Desulfitobacterium* spp. except for *D. hafniense* TCE1 could grow by fumarate disproportionation (data not shown). In the case of nitrate and thiosulfate, an incomplete reduction may explain the high consumption of these substrates. The reasons for this phenomenom are unknown so far. While thiosulfate and sulfite reductase both seem to be present in the genomes of *Desulfitobacterium* spp., differences exist in the genetic setup for nitrate reduction in *D. hafniense* Y51 and DCB-2, which reduce nitrate via a Nar and Nap system, respectively (Nonaka et al., 2006; Kim et al., 2012). Both systems allow for the complete reduction of nitrate to ammonium. An intermediary accumulation of nitrite could, however, be observed in all *Desulfitobacterium* pure cultures with the exception of *D. hafniense* DCB-2. In the case of Fe(III), only half of the predicted amount of electron acceptor was consumed. Since Fe(III) was supplied as Fe(III)citrate, it is possible that citrate might have influenced the stoichiometry of the reaction. This assumption is supported by the fact that a citrate lyase was identified in the genome of *D. hafniense* DCB-2, providing a possibility to utilize this compound or its conversion product oxaloacetate as electron acceptors (Kim et al., 2012). *Desulfitobacterium* spp. might thus also couple the oxidation of the methyl moiety to the reduction of oxaloacetate (as product of citrate cleavage) to succinate. The facts that *D. hafniense* DCB-2 was able to grow on 4-hydroxyanisole and sodium citrate via *O*-demethylation of 4-hydroxyanisole to hydroquinone, as well as on citrate as sole growth substrate, support this theory (data not shown).

3.2 Genetic background of *O*-demethylation in *Desulfitobacterium* spp.

Bacterial *O*-demethylases consist of four different proteins: two methyltransferases (MT I and MT II), a corrinoid protein (CP) and an activating enzyme (AE) (see 1.1.2). The genes encoding for MT I, MT II and CP are usually organized into an operon or cluster, while the gene encoding for AE can be, but is not necessarily, located in the vicinity (Schilhabel et al., 2009). In a previous study it was revealed that the genome of *D. hafniense* DCB-2 harbored up to 17 putative demethylase systems, although it was not possible to discriminate between *O*-, *S*-, *N*-, or *Cl*-demethylases (Studenik et al., 2012). In this study, the amino acid sequences of the four components of the *O*-demethylase of *D. hafniense* DCB-2 characterized by Studenik et al. (2012) were used as templates for BLAST analyses to identify similar demethylase systems in the genomes of *D. dehalogenans*, *D. dichloroeliminans*, *D. metallireducens*, *Desulfitobacterium* sp. LBE, *Desulfitobacterium* sp. PCE1 and *D. hafniense* strains DP7, PCE-S, PCP-1, TCE1, TCP-A and Y51 (see 2.5.1).

With the exception of *D. metallireducens*, putative demethylase systems could be identified in the genomes of all *Desulfitobacterium* species screened (see **Table 3.2**). The highest amount of putative demethylase systems could be identified in the genomes of *D. hafniense* strains DCB-2 (for a physical gene map of putative demethylases, see Studenik et al., 2012) and DP7. **Figure 3.3** shows a physical gene map of putative demethylases in *D. hafniense* DP7. As shown in the figure, a total of 20 CP (orange, length ≈ 600 bp), 12 MT II (blue, length ≈ 800 bp), 17 MT I (yellow, length ≈ 1000 bp) and 6 COG3894-encoding genes (green, variable in length) could be identified, organized into 17 operons. An operon was defined as a group of at least of two components out of CP, MT I and MT II (Studenik et al., 2012). The genes encoding for COG3894 proteins (and thus potentially AE) are usually located in the vicinity of *O*-demethylase operons. The high amount of MT I-encoding genes suggests that a variety of methoxylated aromatics might be utilized by strain DP7, since the substrate specificity of *O*-demethylases is determined by the MT I (Engelmann et al., 2001; Schilhabel et al., 2009; Kreher et al., 2010). As in the case of *D. hafniense* DCB-2, a discrimination between *O*-, *N*-, *S*- and *Cl*-demethylases was not possible for strain DP7 or for any of the other *Desulfitobacterium* species and strains screened. Gene maps for the remaining sequenced *Desulfitobacterium* species can be found in the APPENDIX section (**Figure 6.4**–**Figure 6.11**).

Table 3.2: Number of genes putatively encoding protein components of *O*-, *N*-, *S*-, and *Cl*-demethylases in *Desulfitobacterium* spp. and number of putative demethylase gene clusters. Abbreviations: CP (corrinoid protein), MT I/II (methyltransferase I/II), COG3894 (cluster of orthologous groups 3894). Adapted from Mingo et al. (2014).

Species and strain	Genes Encoding CP	Genes Encoding MT II	Genes Encoding MT I	Genes Encoding COG3894	Number of putative gene clusters[a]
D. hafniense DCB-2	23	14	19	6	18[b]
D. hafniense DP7	20	12	17	6	15
D. hafniense PCE-S	19	11	15	6	15
D. hafniense PCP-1	21	13	17	6	16
D. hafniense TCE1	16	9	13	5	11
D. hafniense TCP-A	14	9	12	5	11
D. hafniense Y51	15	9	10	5	12
Desulfitobacterium sp. LBE	18	9	15	6	13
Desulfitobacterium sp. PCE1	10	5	7	6	7
D. dehalogenans	10	5	7	6	7
D. dichloroeliminans	4	4	4	2	4
D. metallireducens	0	1	0	0	0

[a] A gene cluster is defined as operon with at least two of the following components: CP, MT I, MT II.

[b] Previous studies revealed up to 17 operons (Studenik et al., 2012). In this study another operon was identified.

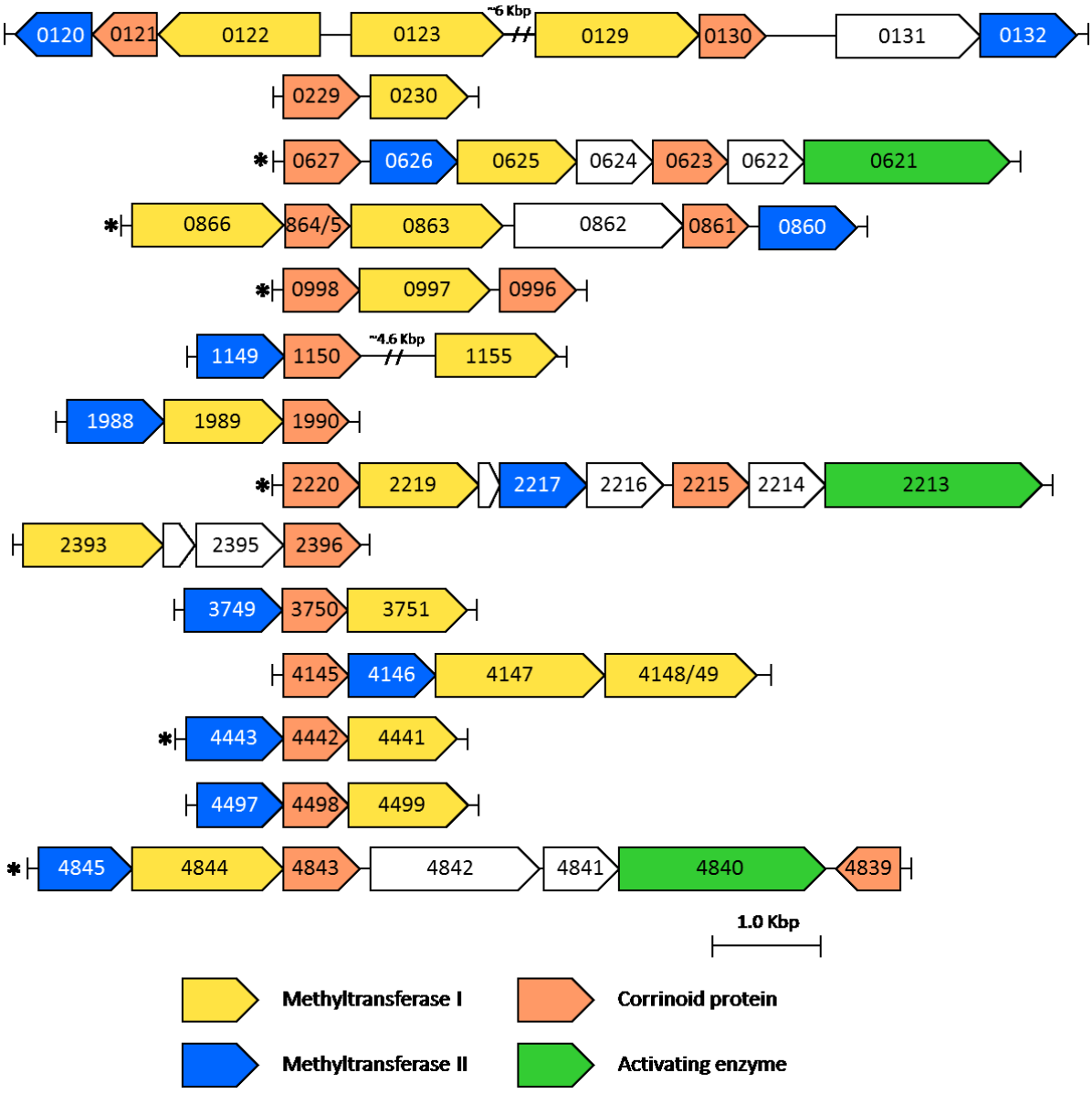


Figure 3.3: Physical gene map of putative demethylase systems in *D. hafniense* DP7. The corresponding genes were identified via the IMG server. Each locus tag number is preceded by the prefix "HMPREF0322_". Asterisks indicate an inverse orientation of all genes included in the cluster compared to the IMG output.

As stated above, the highest numbers of putative demethylase systems were identified in strains of *D. hafniense*, although a high variability could be observed among them. With respect to the numbers of putative demethylase systems (from highest to lowest), *D. hafniense* strains were followed by *Desulfitobacterium* sp. LBE, a species the genome of which shows high similarity to that of *D. hafniense* TCP-A (Tobias Goris, personal communication). *D. dehalogenans* and *Desulfitobacterium* sp. PCE1, which also have similar genomes (Tobias Goris, personal communication), were followed by *D. dichloroeliminans* with the least amount of putative demethylases (see **Table 3.2**).

To check which of the demethylase gene clusters previously identified in *D. hafniense* DCB-2 were conserved among desulfitobacteria, the available genome sequences were analyzed (see **Table 3.3**). The screening revealed the presence of the previously characterized *O*-demethylase of *D. hafniense* DCB-2 (locus tags Dhaf_4610, 4611 and 4612; Studenik et al., 2012) in each of the *D. hafniense* genomes analyzed. The sequence identity on protein level varied between 99–100% for the different demethylase components. A closely related *O*-demethylase operon with a sequence identity on protein level of 94–97% was also found in the genomes of *D. dehalogenans*, *Desulfitobacterium* sp. PCE1 and *Desulfitobacterium* sp. LBE. Moreover, a related *O*-demethylase in *D. dichloroeliminans* had an identity of 88–94% on the protein level for the corresponding components. The Dhaf_0195/0196 operon, which consists of a CP and a MT I, could also be identified in each of the screened genomes, although in the genome of *D. hafniense* TCE1 the MT I (DeshaDRAFT_1207 and 1209) is interrupted by a transposase domain (DeshaDRAFT_1208). For *D. hafniense* strains and *Desulfitobacterium* sp. LBE, the identity on protein level ranged from 98–100% for the two components. In the case of *D. dehalogenans*, *D. dichloroeliminans* and *Desulfitobacterium* sp. PCE1 the identities were lower (68–91%). Aside from these two putative demethylases, the remaining demethylase systems are mostly exclusive to *D. hafniense* strains. Some of the species and strains harbored demethylases for which no homologous counterparts could be identified in the genome of *D. hafniense* DCB-2. This was the case for *Desulfitobacterium* sp. LBE (DesLBEDRAFT_2233 operon), *Desulfitobacterium* sp. PCE1 (DesPCE1DRAFT_0546 operon), *D. dehalogenans* (Desde_0570 operon), *D. dichloroeliminans* (Desdi_2015 operon) and *D. hafniense* strains DP7 (HMP0322_00121 and 00130 and operons) and PCP-1 (A37YDRAFT_0171 operon).

The characterized AE from *D. hafniense* DCB-2 (Dhaf_2573; Studenik et al., 2012) was also present in every genome screened, except for *D. metallireducens* and *D. hafniense* strains TCE1 and Y51. The identity on protein level ranged from 97–99% for the remaining *D. hafniense* strains. In *D. dehalogenans* and *D. dichloroeliminans*, identities of 81% and 42%, respectively, were obtained.

Table 3.3: Identification of homologous demethylase systems in the genus *Desulfitobacterium* via the top homolog tool of the IMG server. All putative demethylase components (MT I, MT II, CP) were compared with the corresponding homologues in *D. hafniense* DCB-2. The putative demethylase gene clusters are represented by the locus tag of the corrinoid protein of the corresponding operon. The corresponding locus tag prefix is shown below each species' name. The locus tag numbers are listed in the table. Some desulfitobacteria harbor putative demethylases that are not present in *D. hafniense* DCB-2. They are not included in the table. Abbreviations: *Dsf*

D. hafniense							*Dsf* sp.	*D. dehalo-*	*Dsf* sp.	*D. dichloro-*
DCB-2	**DP7**	**PCE-S**	**PCP-1**	**TCE-1**	**TCP-A**	**Y51**	**LBE**	***genans***	**PCE1**	***eliminans***
Dhaf_	**HMPREF0322_**	**DPCES_**	**A37YDRAFT_**	**DeshaDRAFT_**	**DeshafDRAFT_**	**DSY**	**DesLBEDRAFT_**	**Desde_**	**DesPCE1DRAFT_**	**Desdi_**
0195	00229	0345	04849	1206[a]	4841	0249	3164	0170	0160	0153
0539	01150	0687	02644	3294	2758	0588	2806	n.p.	n.p.	n.p.
0720	n.p.	n.p.	03778	n.p.	4350	n.p.	n.p.	n.p.	n.p.	n.p.
1107	n.p.	n.p.	01451	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
1603	00998	4027	00160	4489	n.p.	3746	1775	n.p.	n.p.	n.p.
1729	00864/5	n.p.	00286	2125	n.p.	3647	1654	n.p.	n.p.	n.p.
1827	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
1835	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
1895	n.p.	3822	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
2368	03750	1317	02977	2374	2758	1269	n.p.	n.p.	n.p.	n.p.
2567	00627	1589	03146	n.p.	2556	n.p.	0820	2048	2011	n.p.
2641	01990	1684	03389	2699	1246	1511	0711	n.p.	n.p.	n.p.
2929	02396	1951	05350	2990	0972	1776	0421	2376	2297	n.p.
3886	02220	2936	02353	4844	2477	2721	4753	3334	3230	n.p.
4325	04843	3377	01262	1522	n.p.	3155	4305	3694	3572	n.p.
4611	04442	4944	00982	0545	1748	4701	3887	3947	3842	3329
4641	04498	4999	00928	0594	1727	4755	3837	n.p.	n.p.	2550
4872	04145	5246	03552	0827	1533	4973	3470	n.p.	n.p.	n.p.

[a] In *D. hafniense* TCE1, the MT I adjacent to DeshaDRAFT_1206 is disrupted by a transposase recognition sequence.

3.3 *O*-demethylation as a growth-selective process in anoxic soil enrichment cultures

The results presented above demonstrate that *O*-demethylation of phenyl methyl ethers is a widespread characteristic of the genus *Desulfitobacterium* and hint at their involvement in the demethylation of these compounds in the environment. To prove this assumption, anaerobic bacteria were enriched from five different soil samples by using the *O*-demethylation of a phenyl methyl ether as the growth-selective process. The enrichment cultures were incubated at 28ºC in a water bath shaker during five sub-cultivation steps. Genomic DNA that was isolated from every culture was then used to check for the presence of desulfitobacteria via quantitative PCR (qPCR), as well as for the characterization of microbial communities via a phylotype diversity analysis.

3.3.1 Properties of soils sampled for enrichment

For the enrichment of methylotrophic bacteria, soils that represent different stages of a typical pedogenic development series in Central Europe were sampled: a cambisol, a luvisol, a gleysol, a pelosol and a podsol (Blume et al., 2010). The approximate locations of the sampling sites are shown in **Figure 2.1**.The development of each soil was influenced by a different process of pedogenesis. Each soil thus provided a different type of ecological niche that might fit the lifestyle of *Desulfitobacterium* spp. and of methylotrophic bacteria in general. The soils ranged from oxic and acidic to semi-anoxic and neutral. Furthermore, the dominant vegetation species was different at each site. **Table 3.4** summarizes the properties of the sampled soils.

Table 3.4: Physico-chemical and pedogenic properties of the sampled soils. Meaning of soil horizons: A (topsoil), B (subsoil), C (bedrock), G (groundwater-saturated horizon), P (peat horizon), e (eluvial), h (humic), l (lessivage), o (oxidation), r (reduction), s (precipitation of sesquioxides), t (accumulation of clay minerals), w (weathering).

Soil	Location	Succession of soil horizons	Dominant pedogenic process	Influencing soil-forming factors	Vegetation form	pH of topsoil	Aeration and saturation at time of sampling
Cambisol	50°51'57.12"N 11°43'0.11"E Stadtroda	Ah, Bw, C	Formation of clay minerals	Biota, climate, parent material, time	Deciduous forest	4.2	Oxic, dry
Luvisol	50°59'33.64" N11°42'39.97"E Tautenburg	Ah, Al, Bt, C	Lessivage (leaching of clay minerals)	Climate, time, water	Deciduous forest	5.9	Oxic, dry
Gleysol	50°54'54.18"N 11°51'20.83"E Bad Klosterlausnitz	Ah Go, Gr	Gleyification (groundwater saturation)	Climate, time, topography, water	Coniferous forest	3.0	Oxic, dry
Pelosol	50°55'50.11"N 11°37'39.28"E Jena	Ah, P, C	None (climax stadium)	Parent material, time	Non-arable grassland	7.0	Semi-anoxic, saturated
Podsol	50°47'34.78"N 11°39'42.16"E Trockenborn-Wolfersdorf	A(e)h, Ae, B(h)s, B(s)h, C	Podsolization (leaching of aluminum and iron sesquioxides)	Climate, parent material, time, water	Coniferous forest	2.7	Oxic, dry

3.3.2 *O*-demethylation of phenyl methyl ethers by soil enrichments

To ascertain which phenyl methyl ether among 4-hydroxyanisole, syringate, vanillate and isovanillate might be best suited for the enrichment of methylotrophic bacteria in all sampled soils, growth media containing the corresponding phenyl methyl ether (4 mM) and thiosulfate (4 mM) as the electron acceptor were inoculated with the different soil samples (see 2.2.3). The first sub-cultivation served as a pre-culture to select for *O*-demethylating bacteria. The consumption of substrates was monitored during the second sub-cultivation (see 2.3.2). The results are summarized in **Table 3.5**. The native soil microbial communities of all samples were capable of *O*-demethylating at least two of the provided phenyl methyl ethers. All microcosms demethylated syringate and isovanillate. The demethylation of vanillate did not occur in the podsol microcosm, while the demethylation of 4-hydroxyanisole was restricted to the cambisol and gleysol microcosms. No notable differences could be observed in the degradation of the monomethoxylated phenyl methyl ethers compared to syringate. However, the degradation rate of vanillate and isovanillate deviated stronger between the different microcosms compared to the demethylation rate of syringate. The podsol microcosm showed the lowest demethylation rates. The results point to an ecological relevance of the process of *O*-demethylation at all sampling sites. The demethylation rates obtained resembled the ones obtained for *Desulfitobacterium* spp. in pure cultures. Since the degradation rate of syringate fluctuated less among the different microcosms, it was chosen as main electron donor for the upcoming enrichment experiments.

Table 3.5: Demethylation rates of phenyl methyl ethers (µM h^{-1}) by different soil microcosms in the presence of thiosulfate. Both substrates were supplied at an initial concentration of 4 mM. Abbreviation: n.d.o. (no demethylation observed).

Microcosm	4-Hydroxyanisole	Syringate	Vanillate	Isovanillate
Cambisol	21.4	19.5	54.6	68.1
Luvisol	n.d.o.	24.4	23.0	32.3
Gleysol	48.5	16.0	10.8	2.50
Pelosol	n.d.o.	22.5	26.3	53.6
Podsol	n.d.o.	0.90	n.d.o.	2.80

3.3.3 *O*-demethylation of syringate coupled to the reduction of thiosulfate

The *O*-demethylation of syringate was chosen as the growth-selective process for enrichment (see above) and was coupled to the reduction of thiosulfate, a compound that naturally occurs in soils. The pathway of phenyl methyl ether conversion with thiosulfate is depicted in **Figure 3.4**.

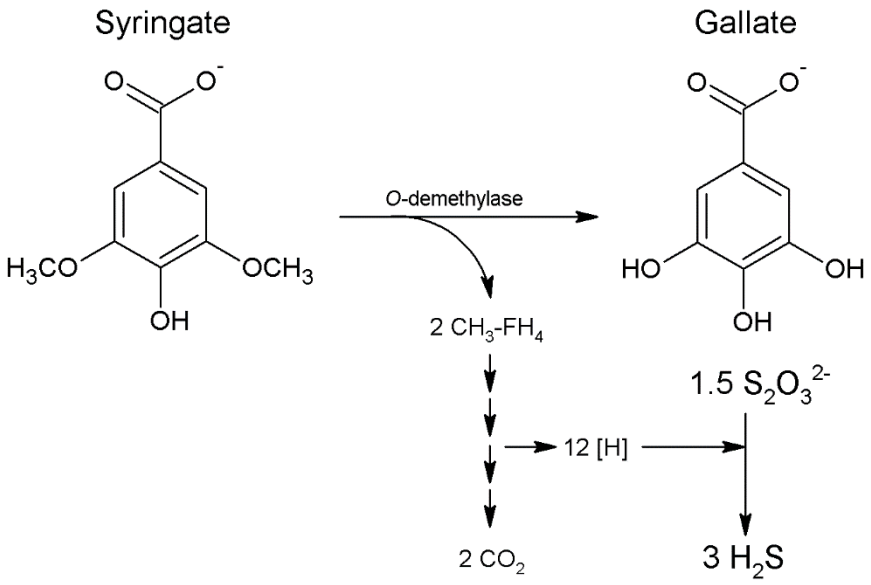


Figure 3.4: *O*-demethylation of syringate coupled to the reduction of thiosulfate to sulfide. Twelve reducing equivalents are derived from the oxidation of the two methyl moieties of syringate to carbon dioxide. In theory, the stoichiometry of thiosulfate consumed per mol methyl moiety is 0.75.

In general, there was no lag prior to the demethylation of syringate. Syringate was demethylated to gallate (3,4,5-trihydroxybenzoate) via 3-*O*-methylgallate (3,4-dihydroxy-5-methoxybenzoate). This process was coupled to the reduction of thiosulfate to sulfide as indicated by the development of a black metal sulfide precipitate in all enrichments. The conversion rate of syringate depended on the enrichment culture and on the sub-cultivation step and differed between the biological duplicates (**Table 3.6**). In general, the conversion rate increased in all enrichment cultures except for the podsol enrichment, which overall showed the lowest conversion rates. The final concentration of gallate did not reach the initial concentration of syringate, suggesting the presence of microorganisms capable of degrading the demethylation products.

Table 3.6: Demethylation rates of syringate (µM h^{-1}) in the presence of thiosulfate. The values are presented as the mean ± standard deviation of the biological duplicates. Abbreviations: n.d. (not determined).

	Rate (µM h^{-1}) in sub-cultivation step				
Enrichment culture	**1**	**2**	**3**	**4**	**5**
Cambisol	26.5 ± 0.5	60.5 ± 22.5	59.0 ± 18.0	82.0 ± 1.0	131.0 ± 0.0
Luvisol	31.5 ± 2.5	31.0 ± 1.0	32.0 ± 1.0	27.5 ± 13.5	80.0 ± 26.0
Gleysol	29.0 ± 7.0	35.5 ± 15.5	67.5 ± 9.5	39.0 ± 0.0	49.0 ± 21.0
Pelosol	35.5 ± 2.5	79.0 ± 4.0	80.0 ± 1.0	81.0 ± 2.0	76.5 ± 6.5
Podsol	15.0 ± 4.0	45.5 ± 29.5	10.0 ± 9.0	n.d.	n.d.

The amount of thiosulfate expected to be consumed per mol methyl group is 0.75 mol (see 3.1). Given the two methoxy moieties of syringate, a consumption of 1.5 mol thiosulfate is expected per mol syringate. The expected stoichiometry was not attained in the enrichment cultures: the amount of thiosulfate consumed per mol syringate was typically lower by a factor of two during the first sub-cultivation and decreased continuously through the later sub-cultivation steps (**Table 3.7**). A non-stoichiometric consumption can be explained by a co-enrichment of acetogenic bacteria in the carbonate-buffered growth medium, since these organisms are able to couple the *O*-demethylation of methoxylated aromatics to CO_2 reduction.

Table 3.7: Consumption of mol thiosulfate per mol syringate in enrichment cultures. The values are presented as the mean ± standard deviation of the biological duplicates. Abbreviations: n.d. (not determined).

	Mol thiosulfate consumed per mol syringate in sub-cultivation step				
Enrichment culture	**1**	**2**	**3**	**4**	**5**
Cambisol	0.94 ± 0.03	0.38 ± 0.08	0.23 ± 0.04	0.09 ± 0.3	0.12 ± 0.07
Luvisol	0.73 ± 0.09	0.65 ± 0.12	0.20 ± 0.12	0.41 ± 0.37	0.09 ± 0.03
Gleysol	0.60 ± 0.19	0.47 ± 0.09	0.16 ± 0.10	0.27 ± 0.11	0.31 ± 0.16
Pelosol	0.77 ± 0.05	0.34 ± 0.12	0.16 ± 0.06	0.26 ± 0.08	0.06 ± 0.06
Podsol	0.55 ± 0.32	0.12 ± 0.05	0.06 ± 0.04	n.d.	n.d.

All enriched consortia were able to grow by the *O*-demethylation of syringate (**Figure 3.5 A–E**) and formed acetate as an end product that was secreted into the media (data not shown). The growth was similar in the different enrichments and typically ended at approximately 100 µg protein/ml culture. The final protein concentration in the enrichments was similar to that of a pure culture (see for example **Figure 3.1**). The enrichment cultures were typically dominated by (sometimes spore-forming) rod-shaped bacteria as observed under a microscope.

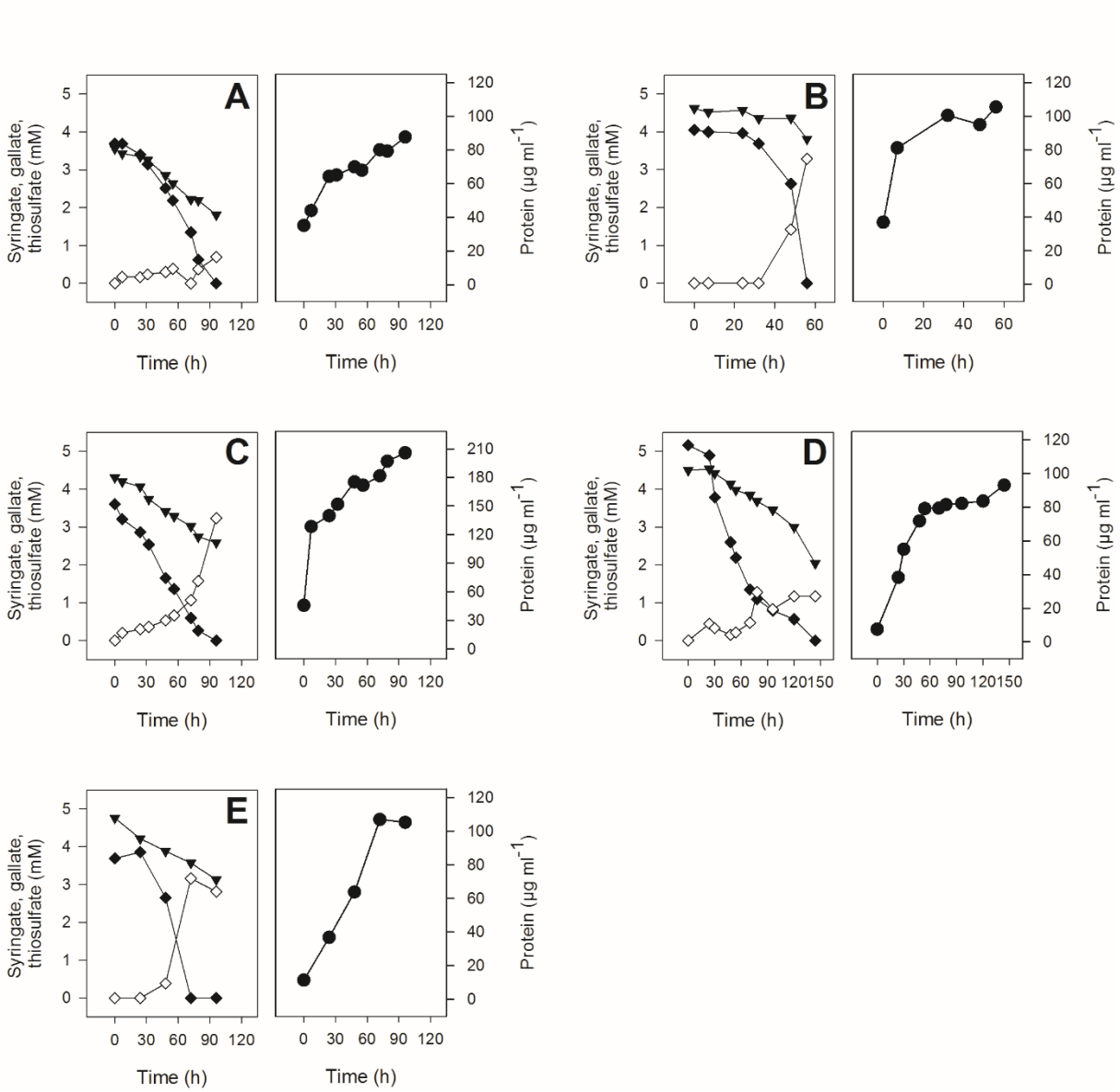


Figure 3.5: Growth curves of cambisol (A), luvisol (B), gleysol (C), pelosol (D) and podsol (E) enrichment cultures (second sub-cultivation). The left panel displays the consumption of growth substrates (closed diamond: syringate; open diamond: gallate; inversed triangle: thiosulfate). The right panel displays the protein concentration.

3.3.4 *O*-demethylation of syringate with chlorinated phenols in cambisol enrichments

Desulfitobacterium spp. are typically described as reductively dehalogenating bacteria. Coupling their methylotrophic metabolism to reductive dehalogenation might provide them with an advantage over co-enriched species that are not capable of this process, given its higher specificity compared to thiosulfate reduction. Thus, the enrichment of *Desulfitobacterium* spp. was also attempted with chlorinated phenols as electron acceptors. The cambisol was chosen as test soil. For the enrichment, 3-chloro-4-hydroxyphenylacetic acid (Cl-OHPA, 5 mM) and 2,4,6-trichlorophenol (TCP, 80 µM) were selected. The dehalogenation of the non-toxic compound Cl-OHPA has been described for some desulfitobacteria (*D. chlororespirans*, *D.*

dehalogenans, *D. hafniense* DCB-2 and *D. metallireducens*), while the dehalogenation of 2,4,6-TCP is more restricted to species that are able to dechlorinate pentachlorophenol (Villemur et al., 2006), but provides an opportunity for *ortho*- and *para*- dechlorinators to grow. In the case of 2,4,6-TCP, the concentration of syringate was adjusted to 500 µM. An additional control enrichment was performed in the absence of an electron acceptor (solely with syringate). Growth was observed in all cutures with concomitant production of acetate (data not shown). For Cl-OHPA, the amount consumed per mol methyl group has been predicted to be 3 mol (Neuman et al., 2004). The observed consumption differed from the predicted one and ranged between 0.04–0.26 mol Cl-OHPA per mol syringate (**Figure 3.6 A**). This can be explained by the co-enrichment of bacteria that are not able to use Cl-OHPA as terminal electron acceptor (e.g. acetogens). No OHPA (4-hydroxyphenylacetic acid) could be detected via HPLC, indicating that the aromatic ring of Cl-OHPA is cleaved before or immediately after dehalogenation. In the case of 2,4,6-TCP, the low concentration applied led to the preparation of a low dilution of samples for signal detection via HPLC, in which the signal of syringate interfered with the detection of TCP. Therefore, only the consumption of syringate was recorded. Syringate was completely demethylated upon consumption of 80 µM 2,4,6-TCP. For the removal of one chlorine substituent, 2 reducing equivalents are needed. Since the oxidation of the methyl moiety of CH_3-FH_4 generates 6 reducing equivalents, three chlorine substituents can be removed with one methyl group of syringate. Therefore, 2 mol 2,4,6-TCP should be consumed per mol syringate. In the control enrichment, no additional electron acceptor was provided except for CO_2. In this culture, the demethylation of syringate was obviously coupled to the reduction of CO_2, which is provided as HCO_3^- in the growth medium (**Figure 3.6 B**). Three mol of CO_2 are expected to be consumed per mol methyl group. Microorganisms that couple the *O*-demethylation of phenyl methyl ethers to the reduction of an alternate electron acceptor (e.g. *Desulfitobacterium* spp.) are discriminated by this enrichment strategy. No significant differences in the growth yields were observed between the control enrichment and the enrichment culture amended with Cl-OHPA. The final protein concentration in the enrichment culture amended with 2,4,6-TCP was three times lower than in the culture amended with Cl-OHPA (data not shown). The lower protein concentration can be explained by the lower substrate concentrations that were applied in the case of the TCP enrichment culture.

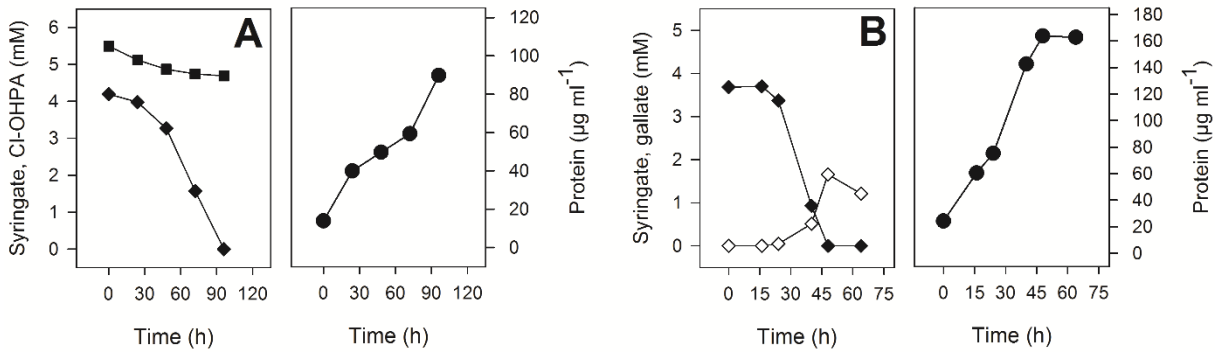


Figure 3.6: Growth of cambisol enrichment cultures with syringate and Cl-OHPA (A) and in the absence of an electron acceptor (B). The left panel displays the consumption of growth substrates (closed diamond: syringate; open diamond: gallate, cube: Cl-OHPA). The right panel displays the protein concentration. No gallate could be detected during the cultivation with Cl-OHPA

3.4 Enrichment of *Desulfitobacterium* spp. from soils

After syringate could no longer be detected via HPLC, genomic DNA was extracted from the enrichment cultures. The enrichment cultures were then transferred to fresh growth medium (10% [v/v] inoculum). Five sub-cultivation steps were performed in total for every enrichment culture except for the podsol enrichment cultures due to low conversion rates. The detection of *Desulfitobacterium* spp. was then attempted via two different approaches, namely quantitative PCR (qPCR) and fluorescence *in situ* hybridization (FISH). The qPCR assay was applied to each enrichment culture, while FISH experiments were performed occasionally and served as further verification of the results obtained via qPCR.

3.4.1 Enrichment cultures amended with syringate and thiosulfate

Genomic DNA from soils and enrichment cultures amended with syringate and thiosulfate was analyzed for the presence of *Desulfitobacterium* spp. 16S rRNA gene copies by the use of a genus-specific primer pair. The quantification showed a consistent trend in all enrichment cultures: While the relative amount of *Desulfitobacterium* spp. 16S rRNA gene copies did not differ much between the sampled soils, an increase in the gene copy number was observed in all cultures amended with syringate and thiosulfate (**Figure 3.7**).

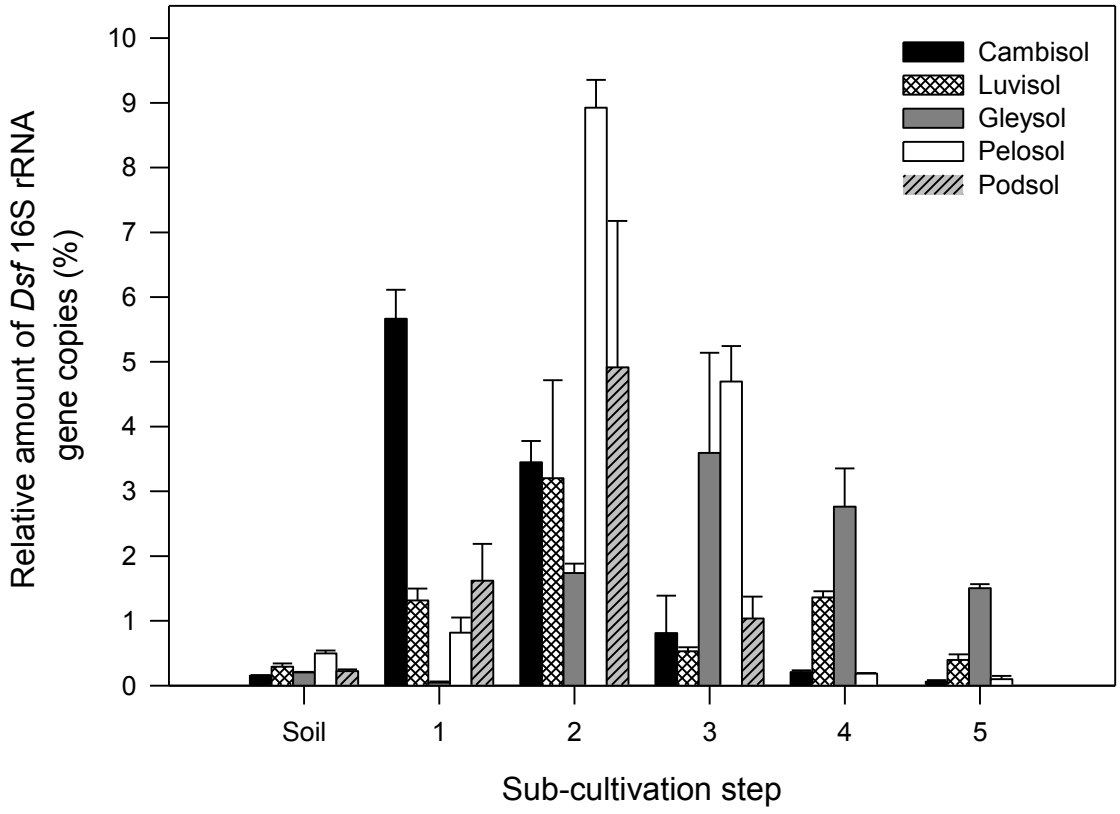


Figure 3.7: Detection of *Desulfitobacterium* spp. 16S rRNA gene copies by qPCR in enrichment cultures amended with syringate and thiosulfate. For each enrichment only one of the biological duplicates is shown. The data are presented as mean ± standard deviation of technical triplicates. Abbreviations: *Dsf* (*Desulfitobacterium* spp.).

A maximum was usually reached at the end of the second sub-cultivation step, although it was sometimes shifted to the end of the first or third sub-cultivation. With the exception of podsol enrichment cultures, the maximum relative gene copy number enriched did not differ considerably between the biological duplicates of each enrichment (see APPENDIX section, **Table 6.1**). The increase was followed by a decline in the relative gene copy number throughout the later sub-cultivation steps, often reaching relative amounts of 1% or less at the end of the fifth sub-cultivation. The initial amount of *Desulfitobacterium* spp. (from now on abbreviated *Dsf*) present in soils as well as the maximum amount enriched from each soil are summarized in **Table 3.8**.

Table 3.8: Properties of sampled soils and desulfitobacteria enriched.

Soil	*Desulfitobacterium* spp. 16S rRNA gene copies in topsoil (%)	Highest proportion enriched (%)[a]	Identified species in 2nd sub-cultivation
Cambisol	0.15	8.2	*D. hafniense* DCB-2 (97)[b] *D. chlororespirans* (98) *D. dehalogenans* (96)
Luvisol	0.29	3.5	*D. dichloroeliminans* (95)
Gleysol	0.20	10.0	*D. hafniense* DCB-2 (99) *D. dichloroeliminans* (97)
Pelosol	0.49	8.9	*D. hafniense* DCB-2 (99) *D. dichloroeliminans* (97) *D. metallireducens* (97)
Podsol	0.22	4.9	*D. hafniense* DCB-2 (99)

[a] Applies to enrichments with syringate as electron donor and thiosulfate as electron acceptor. [b] The value in parenthesis represents the percent identity with known *Desulfitobacterium* 16S rRNA sequences obtained from BLAST N alignments.

The enrichment with syringate and thiosulfate led to an approximately tenfold increase in *Dsf* gene copies in the luvisol, twentyfold in the pelosol and podsol and fiftyfold in the cambisol and gleysol cultures. No relationship could be found between the relative gene copy number enriched from each topsoil and its initial pH, demonstrating that *Desulfitobacterium* spp. can be enriched successfully from acidic to neutral soils. Cloning and sequencing of the qPCR products of the second sub-cultivation step showed that in four out of five enrichments, sequences with a high similarity to *D. hafniense* DCB-2 were present (see **Table 3.8**). Sequences related to *D. chlororespirans*, *D. dehalogenans*, *D. dichloroeliminans* and *D. metallireducens* were also recovered. By definition, organisms that share an identity of ≥ 97% in their 16S rRNA gene sequences are classified as members of the same species (Stackenbrandt & Goebel, 1994). Most of the cloned sequences can thus be considered *Dsf* sequences.

The presence of *Desulfitobacterium* spp. in enrichment cultures was also verified via FISH. For this, genus-specific probes were used (Yang et al., 2005). *Desulfitobacterium* spp. appeared rod-shaped, consistent with the morphology of *D. hafniense* DCB-2, which was used as the positive control (**Figure 3.8**). No efforts of cell counting were undergone, as the relative cell count depended on the spatial distribution of cells in the wells of microscopy slides. As stated before, the enrichments were dominated by rod-shaped bacteria, although in some cases coccoid bacteria could also be observed by DAPI stain.

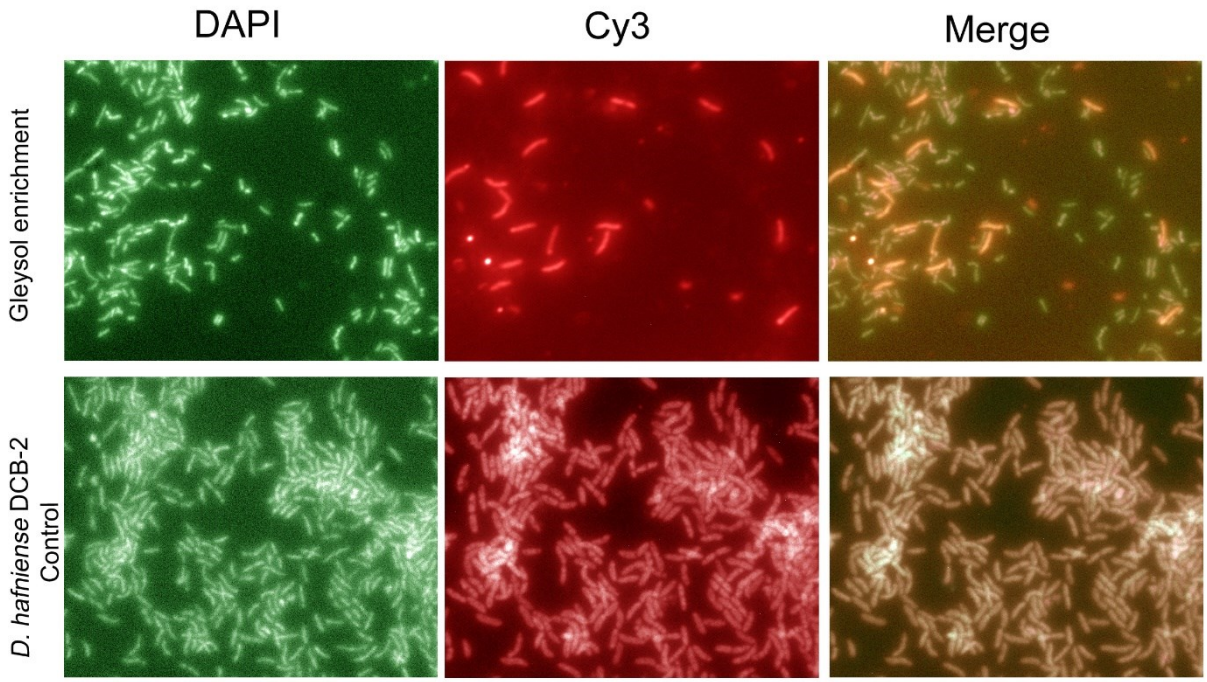


Figure 3.8: Epifluorescence image of a gleysol enrichment culture amended with syringate and thiosulfate, during the second sub-cultivation. Left panel: DNA stain (DAPI); middle panel: *Desulfitobacterium* spp. 16S rRNA stain (Cy3-labelled probe); right panel: overlay/merge.

In soils, the detection of *Desulfitobacterium* spp. was hampered by low cell densities and fluorescence signals generated by inorganic artifacts (e.g. clay minerals). Substituting the detection on microscopy slides by the detection on top of agarose-coated membrane filters failed to yield any improvements.

3.4.2 Enrichment cultures amended with syringate and chlorinated phenols

The enrichment of *Desulfitobacterium* spp. from cambisol was also attempted with alternate electron acceptors. For this, the *O*-demethylation of syringate was coupled to the reductive dechlorination of Cl-OHPA or 2,4,6-TCP. An additional enrichment that lacked an electron acceptor was also transferred for five sub-cultivations. These experiments were performed with unique enrichment culture replicates. The quantification via qPCR showed a similar trend as described before for enrichments with the syringate/thiosulfate couple (**Figure 3.9**).

No evident enrichment of desulfitobacteria could be observed in the culture amended with 2,4,6-TCP. This can be explained by the low applicable concentration of 2,4,6-TCP due to the toxicity of this compound and its metabolites. Constraints in the applicable concentration of similar organohalides for the enrichment of reductively dechlorinating bacteria have been described before (Lechner, 2007). The applied concentration of 80 µmol/l represent the maximal concentration that can be tolerated by *D. hafniense* DCB-2 (Anita Mac Nelly, personal

communication). Assuming that *Desulfitobacterium* spp. were the only responsible organisms for the dechlorination of this compound, 40 µmol/l syringate could be coupled to the dechlorination of 80 µmol/l 2,4,6-TCP. This represents a strongly limiting quantity of growth substrates and might be the cause for the poor enrichment yield. Nevertheless, *Desulfitobacterium*-specific signals could be detected in all sub-cultivations, proving the ability of these bacteria to resist complete out-competition by co-enriched species.

The enrichment with Cl-OHPA led to an increase of *Desulfitobacterium* spp. 16S rRNA gene copies up to 1% of the total 16S rRNA gene copy number (tenfold increase). Surprisingly, this was also the case for the control enrichment. Given the fact that *Desulfitobacterium* spp. is unable to use CO_2 as terminal electron acceptor (Neumann et al., 2004; Kreher et al., 2008), a possible explanation for their enrichment is a trophic link with co-enriched microorganisms. The underlying mechanisms or interactions remain unknown.

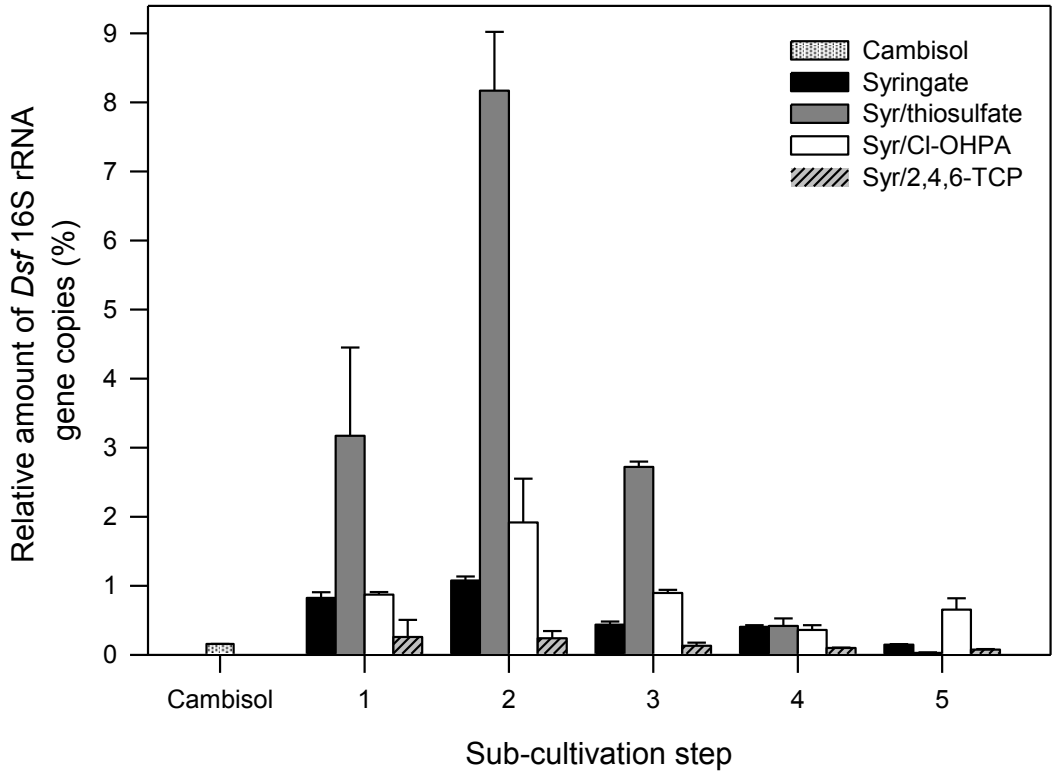


Figure 3.9: Comparison of relative *Dsf* 16S rRNA gene copy numbers enriched from the starting material (cambisol) in the presence of syringate and different electron acceptors. The data are presented as mean ± standard deviation of technical triplicates. Abbreviations: *Dsf* (*Desulfitobacterium* spp.), Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), TCP (trichlorophenol).

While the relative gene copy number decreased to ≤ 0.2% in the control enrichment after reaching its maximum (1%), the gene copy number seemed to reach a plateau in the culture amended with Cl-OHPA. To verify this observation, the Cl-OHPA enrichment was transferred another five times until sub-cultivation step ten. The course of 16S rRNA gene copy numbers was then determined by qPCR. The relative gene copy number seemed to plateau at approximately 0.3–0.4% of the total 16S rRNA gene copy number. This corresponds to a threefold to fourfold amount of gene copies compared to the relative copy number in soil (see **Figure 3.10**).

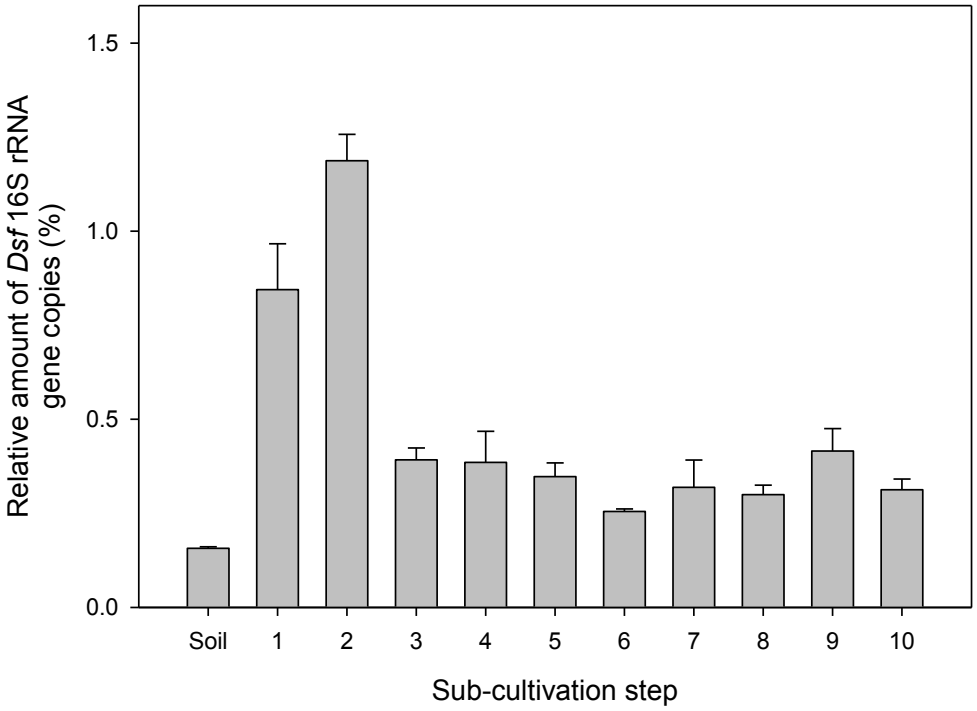


Figure 3.10: Detection of *Desulfitobacterium* spp. 16S rRNA gene copies by qPCR in enrichment cultures amended with syringate and Cl-OHPA. The data are presented as mean ± standard deviation of technical triplicates. Abbreviations: *Dsf* (*Desulfitobacterium* spp.).

From cambisol enrichments it could be concluded that thiosulfate favors a stronger growth compared to other electron acceptors. However, long-term enrichment with Cl-OHPA hints a better survival of *Desulfitobacterium* spp. during longer cultivation periods and points to a possible niche in the environment.

3.5 Formyltetrahydrofolate synthetase as a marker gene for the detection of *Desulfitobacterium* spp. in environmental samples

Formyltetrahydrofolate synthetase (FTHFS) is a key enzyme in the C_1 metabolism of anaerobic methylotrophic organisms and is involved in the reduction of CO_2 to a methyl group in methyltetrahydrofolate (CH_3-FH_4). It catalyzes the reversible ATP-dependent activation of formate and its ligation to the N10 position of tetrahydrofolate (Ragsdale & Pierce, 2008; **Figure 3.11**). In addition, it serves an important role in the biosynthesis of a variety of compounds such as amino acids or purines (Whitehead et al., 1988). Since acetogens comprise a functional group of bacteria that are not necessarily phylogenetically related to each other but show a high homology of their genes encoding FTHFS, this gene has been used as a marker to reveal their presence in several environments (for some examples see Matsui et al., 2008; Xu et al., 2009; Henderson et al., 2010; Lever et al., 2010).

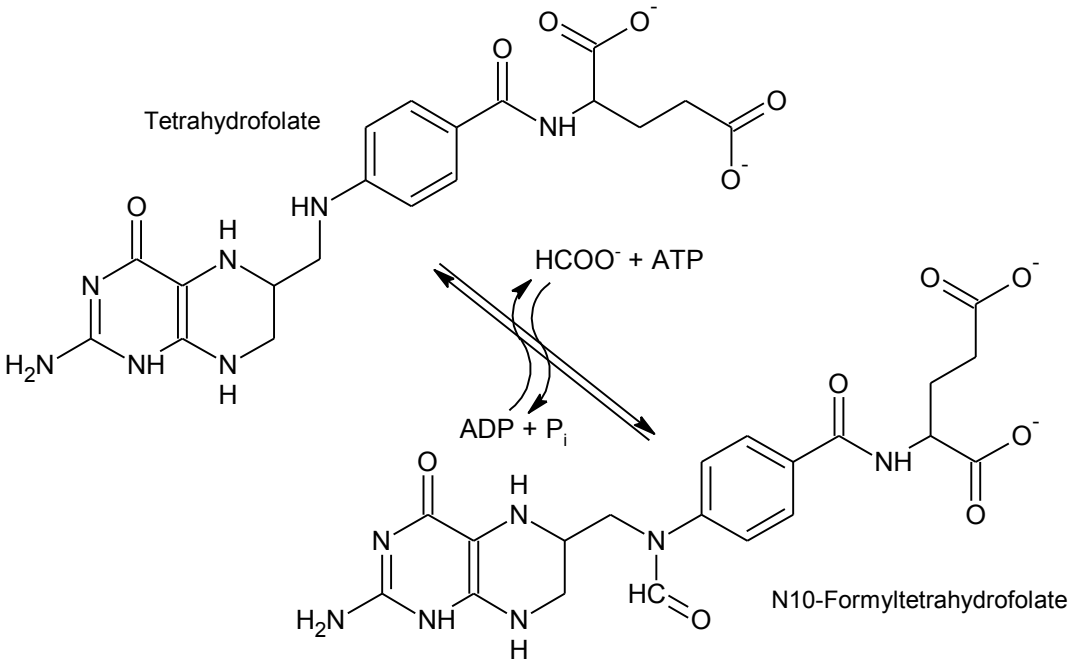


Figure 3.11: Reversible ligation of formate to the N10 position of tetrahydrofolate mediated by formyltetrahydrofolate synthetase.

The qPCR assay based on the detection of 16S rRNA gene copies of *Desulfitobacterium* spp. does not allow for a direct quantification of cell numbers. This is due to a different amount of heterogeneous 16S rRNA gene copies present in each *Desulfitobacterium* sp. genome (Villemur et al., 2007). To determine the cell number of desulfitobacteria in enrichment cultures and soils, and to assess their possible role among other *O*-demethylating co-enriched microorganisms,

FTHFS was chosen as marker gene for the detection of *Desulfitobacterium* spp. in the sampled topsoils and in enrichment cultures.

3.5.1 Genetic background of FTHFS in *Desulfitobacterium* spp.

In *Desulfitobacterium* spp., two FTHFS gene copies were identified in all genomes except for *D. dichloroeliminans* and *D. metallireducens*, in which only one gene copy is present. In *D. hafniense* DCB-2, the corresponding locus tags were Dhaf_0149 (GenBank accession no. NC_011830.1) and Dhaf_0555 (GenBank accession no. NC_011830.1) (**Figure 3.12**).

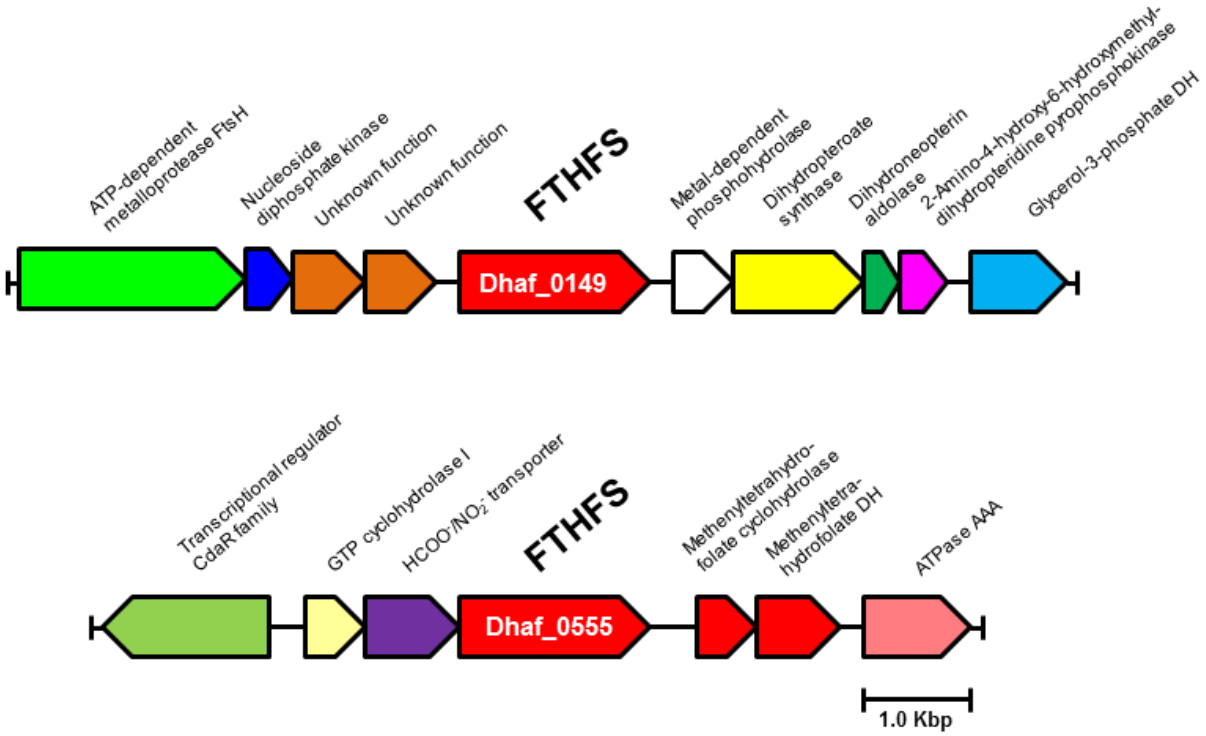


Figure 3.12: Physical gene map showing the location of the two FTHFS gene copies in the genome of *D. hafniense* DCB-2. Abbreviations: DH (dehydrogenase).

The two FTHFS enzymes in *D. hafniense* DCB-2 share an identity of 66% at the amino acid level. A homolog of Dhaf_0149 could be identified in every *Desulfitobacterium* genome sequenced so far, with an identity of at least 87% at the amino acid level. The sequence identity for Dhaf_0555 homologs, which are not present in *D. dichloroeliminans* and *D. metallireducens*, was at least 95% at amino acid level (**Table 3.9**). For those *Desulfitobacterium* species that harbored two FTHFS gene copies in their genome, the sequence identity of both gene products ranged from 65–66% at amino acid level.

Table 3.9: Identification of homologous FTHFS enzymes in *Desulfitobacterium* spp. Pairwise BLAST P alignments were used to identify the homologous counterparts of the two FTHFS enzymes present in *D. hafniense* DCB-2 (Dhaf_0149 and Dhaf_0555). The results are presented in % identity with Dhaf_0149 and Dhaf_0555 at the amino acid level. Abbreviation: n.p. (not present).

Species		Locus tag	Dhaf_0149	Dhaf_0555
D. hafniense	DP7	HMPREF0322_01063	**100**	66
		HMPREF0322_01130	65	**99**
	PCP-1	A37YDRAFT_02627	65	**99**
		A37YDRAFT_03825	**99**	66
	TCE1	DeshaDRAFT_1153	**100**	66
		DeshaDRAFT_3312	65	**99**
	TCP-A	DeshafDRAFT_1280	**100**	66
		DeshafDRAFT_4516	66	**100**
	Y51	DSY0205	**100**	66
		DSY0605	65	**99**
D. dehalogenans		Desde_0117	**97**	66
		Desde_3683	65	**95**
D. dichloroeliminans		Desdi_0081	**92**	n.p.
D. metallireducens		Desme_00390	**87**	n.p.
Desulfitobacterium sp. LBE		DesLBEDRAFT_2788	66	**99**
		DesLBEDRAFT_3221	**99**	66
Desulfitobacterium sp. PCE1		DesPCE1DRAFT_0113	**97**	66
		DesPCE1DRAFT_3561	65	**95**

In this study, two primer pairs, each specifically targeting either Dhaf_0149 or Dhaf_0555 and their corresponding homologs in other *Desulfitobacterium* spp., were designed (see **Table 2.3**). The primer pairs were tested with genomic DNA extracted from *D. chlororespirans*, *D. dehalogenans*, *D. metallireducens* and *D. hafniense* strains DCB-2, DP7, PCE-S, PCP-1, TCE1, TCP-A and Y51. Both primer pairs yielded a single PCR product that was identified as the corresponding FTHFS copy by Sanger sequencing after ligation of the PCR product into the pPrime Cloning Vector (data not shown). The primer pair designed for detection of Dhaf_0555 did not yield any product in *D. metallireducens*, staying in accordance with BLAST P alignments (see **Table 3.9**). To exclude the possibility of mismatch amplification of acetogenic FTHFS, the specificity of both primers was tested with genomic DNA extracted from *Acetobacterium dehalogenans* and *Sporomusa ovata*, two acetogens. No products were generated by both primer pairs (data not shown). The specificity of both primer pairs was further tested in enrichment cultures. The PCR products obtained were purified, cloned into the pPrime cloning vector and transformed in *E. coli* XL1 blue. Plasmids were extracted from clones and

the inserts were sequenced. All sequences were related to *Desulfitobacterium* spp. (data not shown).

3.5.2 FTHFS gene expression in *D. hafniense* DCB-2 during growth with different substrates

To assess the role of both FTHFS gene copies in the methylotrophic metabolism of *Desulfitobacterium* spp., *D. hafniense* DCB-2 was grown under different conditions (pyruvate/fumarate, pyruvate/nitrate, 4-hydroxyanisole/fumarate, 4-hydroxyanisole/nitrate) and the expression of both FTHFS copies was investigated via reverse transcription PCR (RT-PCR, see 2.4.4) after isolation of RNA at various time points of growth (see 2.4.3). The results are displayed in **Figure 3.13**. For the detection of Dhaf_0149 transcripts, a tenth of the total RNA amount used for detection of Dhaf_0555 and rpoB transcripts was used. Therefore, the gel images are not fully comparable. When using 1000 ng total RNA as template for Dhaf_0149, the signal strength of bands was equal for all time points, indicating that the amplification reaction had reached a saturation point. In order to prove an up-regulation of the gene in response to growth conditions, less amounts of template were used for Dhaf_0149.

At the beginning of the cultivation, bands of the target and reference genes were either very weak or could not be detected at all. The band of the reference gene, rpoB (encodes for the beta subunit of the RNA polymerase), showed a mostly uniform strength, and its expression pattern was not affected by the growth conditions. Transcripts of both FTHFS gene copies could be detected under all growth conditions, indicating that both are likely expressed constitutively. An increase in transcript levels of both genes was observed already a couple of hours after inoculation when *D. hafniense* DCB-2 was grown on pyruvate/fumarate or on pyruvate/nitrate (see **Figure 3.13**). In the case of growth on 4-hydroxyanisole/fumarate and on 4-hydroxyanisole/nitrate (**Figure 3.13**), the increase in transcript levels of Dhaf_0149 was observed 24 hours later than during growth with pyruvate. In contrast to this, Dhaf_0555 transcripts sometimes remained undetectable until several hours after the detection of Dhaf_0149 transcripts. The signal bands obtained from the amplification of Dhaf_0149 transcripts were stronger than for Dhaf_0555 transcripts, even though the amount of template used for the detection of Dhaf_0555 was tenfold higher than for Dhaf_0149. These results suggest low transcript levels of Dhaf_0555, whereas Dhaf_0149 seems to be transcribed in larger quantities in response to the growth substrates provided.

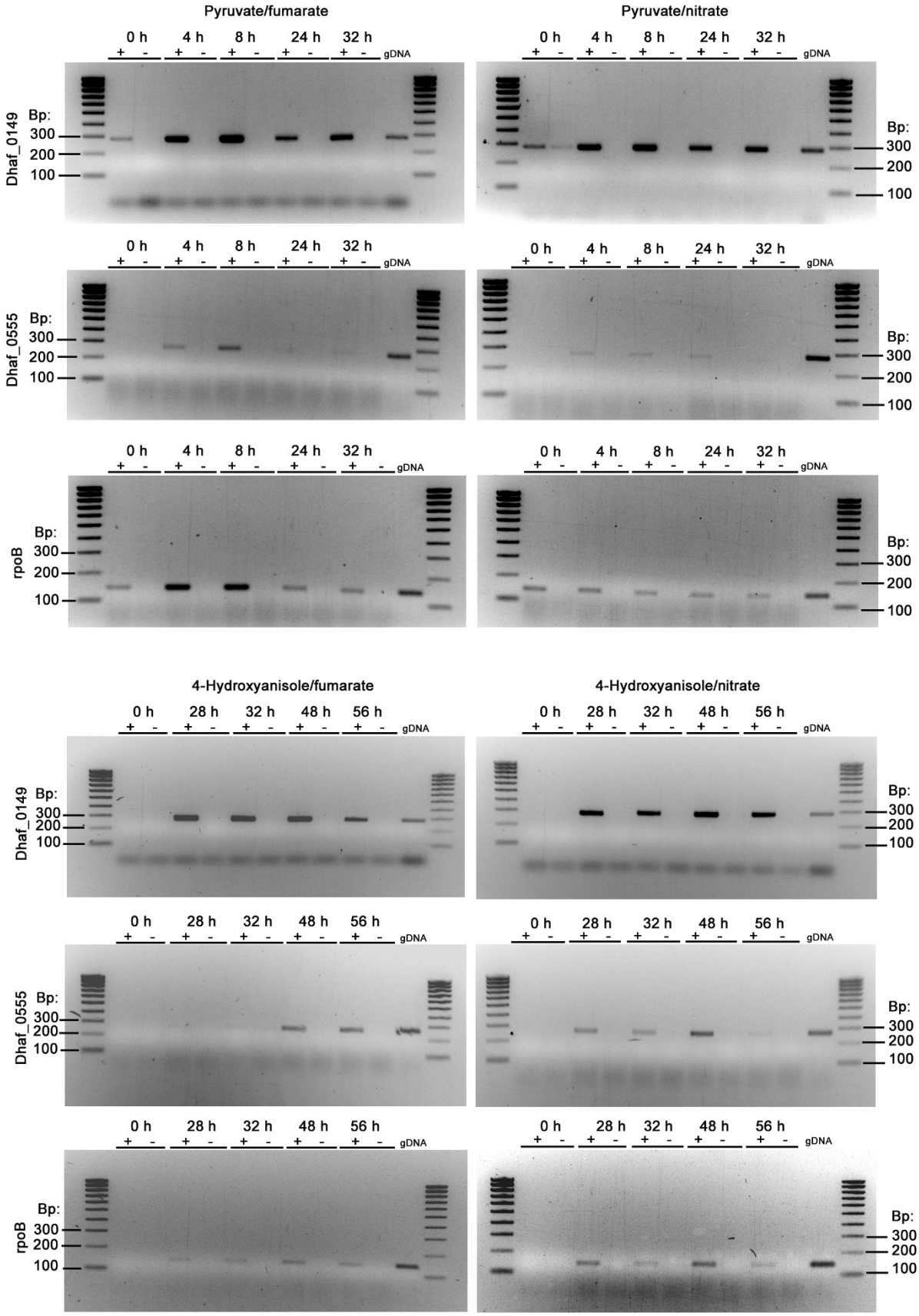


Figure 3.13: FTHFS expression pattern in *D. hafniense* DCB-2 during growth on different substrates. For the detection of Dhaf_0149, 100 ng total RNA were used as template. In the case of Dhaf_0555 and the reference gene, rpoB, 1000 ng total RNA were used as template. Figure legend: + (+RT sample; reverse transcription took place), - (-RT sample, reverse transcription did not take place). h (hours after inoculation).

3.5.3 Detection of FTHFS gene copies in enrichment cultures

RT-PCR experiments revealed the dominance of Dhaf_0149 transcripts over Dhaf_0555 transcripts in *D. hafniense* DCB-2. Additionally, in contrast to Dhaf_0555, a homolog of Dhaf_0149 is present in every *Desulfitobacterium* genome checked (see **Table 3.9**). The Dhaf_0149 copy shares a 60-70% identity at the amino acid level with the FTHFS enzyme of various acetogenic bacteria and features distinct amino acid residues that can be targeted by specific primers at the DNA level (see APPENDIX section, **Figure 6.2**). Hence, Dhaf_0149 and its corresponding homologs in the genomes of other desulfitobacteria were chosen to serve as marker gene for the detection of *Desulfitobacterium* spp. in enrichment cultures via the Dhaf_0149-specific primer set. In order to ascertain the relative amount of *Desulfitobacterium* spp. among co-enriched acetogens, a primer pair published by Xu et al. (2009) was used to determine the gene copy number of genuinely acetogenic FTHFS. However, PCR experiments using genomic DNA of *Desulfitobacterium* spp. as template revealed that also FTHFS gene copies of desulfitobacteria were amplified by this primer pair (data not shown). Since the primer pair is not as specific as previously suggested, its amplification product in enrichment cultures is referred to as "total" FTHFS gene copies (as in total of copies amplified in the enrichment cultures) instead of genuinely acetogenic FTHFS.

With the primer pair described by Xu et al. (2009) it was possible to detect signals in all soils sampled as well as in all enrichment cultures. However, only a rough estimation of the number of acetogens was possible by this method, as many of them also harbor two FTHFS gene copies in their genome (Gagen et al., 2010). With the primer pair designed for specific detection of *Desulfitobacterium* FTHFS (Dhaf_0149 and its homologs in other desulfitobacteria; from now on *Dsf* FTHFS), no signals could be detected in soils. However, its detection was possible in enrichment cultures. **Figure 3.14** shows abundance of *Dsf* FTHFS in syringate/thiosulfate enrichment cultures, quantified via the *Dsf*-specific FTHFS primer pair, expressed as percent of the total FTHFS gene copy number that was quantified via the primer pair of Xu et al. (2009). The relative amount of *Dsf* FTHFS varied significantly between the biological duplicates of an enrichment (see APPENDIX section, **Table 6.1**). The relative *Dsf* FTHFS values were considerably higher in both cambisol duplicates than in the remaining enrichments. One of the pelosol enrichments also showed high relative amounts of *Dsf* FTHFS. To exclude the possibility of a primer mismatch, the qPCR product of the first cambisol sub-cultivation step (see **Figure 3.14**) was cloned and sequenced. The first hit of all analyzed sequences corresponded to *Desulfitobacterium* spp. with an identity of 94–99% as revealed by BLAST N

alignments, the hit for the second organism being *Desulfosporosinus orientis* with an identity of 79%. This clone library, along with a former library that had also been constructed from enrichment cultures to verify the specificity of the primer pair after its design, confirmed the specificity of the primer pair (specific match for all 24 analyzed clones). Thus, the surprisingly high relative *Dsf* FTHFS amounts could indeed be assigned to specific *Dsf* FTHFS signals. One reason for the high values might lay in the above discussed unspecificity of the primer pair designed by Xu et al. (2009), which was initially intended for amplification of "acetogenic" FTHFS, but which also amplifies desulfitobacterial FTHFS gene copies. The entirety of amplified acetogenic and non-acetogenic FTHFS gene copies by the primer pair of Xu et al. (2009) is unknown and needs to be tested with further organisms. The discrimination of acetogens that are highly abundant in the enrichment cultures, but which may not be recognized by the primer pair, might explain the high relative *Dsf* values.

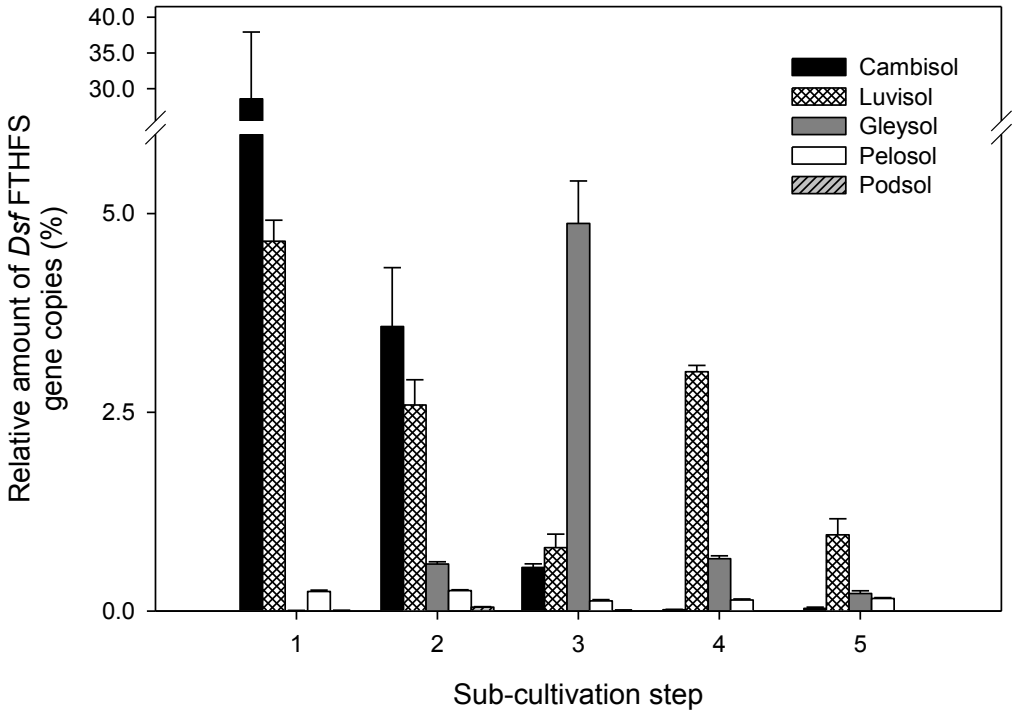


Figure 3.14: Detection of *Desulfitobacterium* spp. FTHFS gene copies by qPCR in enrichment cultures amended with syringate and thiosulfate. Only one of the biological duplicates is shown for each enrichment. The cultures shown are the same as in Figure 3.7. The data are presented as mean ± standard deviation of technical triplicates. Abbreviations: *Dsf* (*Desulfitobacterium* spp.).

In the case of luvisol, gleysol and podsol enrichments, the relative *Dsf* FTHFS values were more similar among biological duplicates, but in some of the cultures they were barely detectable. This might be due to a restriction in the amount of template that can be used in the total FTHFS assay. The primer pair designed for the detection of total FTHFS generates unspecific products if total DNA amounts of more than 2 ng per reaction are used as template

(Xu et al., 2009). To keep the results comparable with 16S rRNA qPCR results, 1 ng gDNA was used as template. The corresponding amount of template used for the detection of *Dsf* FTHFS was therefore the same. Using a higher amount of template would have facilitated the detection of *Dsf* gene copies, but would have interfered with the total FTHFS assay. Therefore, the 16S rRNA qPCR assay offers a higher resolution than the FTHFS assay, since several 16S rRNA gene copies are detected in one genome, while only one FTHFS gene copy is detected per genome in the FTHFS assay.

In cambisol enrichment cultures in which thiosulfate was substituted by Cl-OHPA or 2,4,6-TCP, the relative amount of *Dsf* FTHFS gene copies stayed at low relative quantities. In the enrichment with 2,4,6-TCP, gene copies were not detectable during the second and third sub-cultivation step, but were recovered during the fourth and fifth sub-cultivation. Surprisingly, the control enrichment that lacked an alternate electron acceptor showed the second highest relative amounts of *Dsf* FTHFS gene copies (approximately 16%, **Figure 3.15 A**) after the enrichment on syringate and thiosulfate (approximately 29%, **Figure 3.15 B**). Possible reasons for these high values were already explained above. The detection of *Dsf* FTHFS in the control enrichment that lacks an electron acceptor verifies the 16S rRNA qPCR results obtained for the same culture and demonstrates the possibility of *Desulfitobacterium* spp. to be enriched by providing only a phenyl methyl ether for enrichment at least for a few sub-cultivation steps.

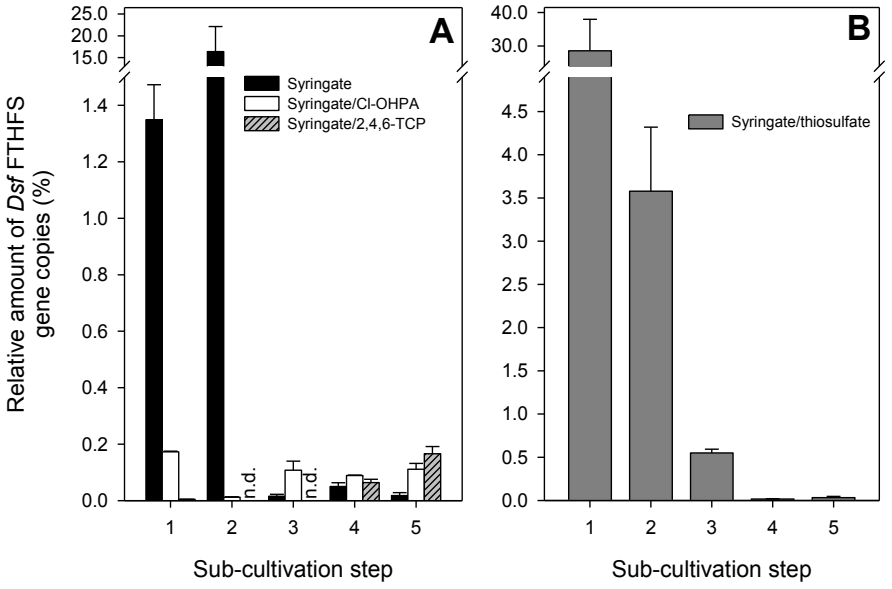


Figure 3.15: Detection of *Desulfitobacterium* spp. FTHFS gene copies by qPCR in cambisol enrichment cultures amended with syringate and chlorophenols (A). The enrichment with syringate and thiosulfate (B) is shown as a reference. The data are presented as mean ± standard deviation of technical triplicates. Abbreviations: *Dsf* (*Desulfitobacterium* spp.), Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), TCP (trichlorophenol), n.d. (not detectable).

3.6 Analysis of soil and enriched microbial communities

The detection of *Desulfitobacterium* spp. in the sampled soils as well as in all enrichment cultures was possible via qPCR. However, after an initial enrichment, which was reflected in an increase of the relative amount of *Desulfitobacterium* spp. 16S rRNA gene copies, a gradual loss was observed. This suggested an out-competition by co-enriched bacteria. In order to elucidate this phenomenon, microbial communities were analyzed via Illumina MiSeq technology. This was done for the five soil microbial communities as well as for two sub-cultivations of each enrichment, namely the sub-cultivation step containing the maximum amount of *Desulfitobacterium* spp. 16S rRNA gene copies (usually the second or third sub-cultivation, and from now on termed intermediate sub-cultivation step) and the last sub-cultivation. In case of those enrichments amended with syringate/thiosulfate, only one replicate was analyzed, corresponding to the one shown in the qPCR sections.

3.6.1 Overall sequencing results

A total amount of 381,637 sequences remained after completion of the pipeline processing and could be classified into operational taxonomic units (OTUs), with an average sequence number of 18,173 per sample. The minimum amount of 3,000 reads warranted by the Research and Testing Laboratory was surpassed in all samples by at least 1,500 sequences after the pipeline process had been completed (**Table 3.10**). The amount of reads was typically highest in soil samples, followed by the final sub-cultivation step of each enrichment and the intermediate sub-cultivation step. This probably reflects differences in the quality of extracted DNA. The diversity-narrowing effect of the enrichment procedure was reflected in the decrease of observed OTUs with advancing enrichment. After five sub-cultivation steps (three sub-cultivation steps for the podsol), the number of native soil species had decreased by an approximate factor of two in the gleysol, three in the podsol, six in the cambisol and luvisol, and ten in the gleysol culture.

Table 3.10: Benchmark data of Illumina MiSeq characterization of microbial communities in soils and enrichment cultures.

Sample	Quality reads	Species observed
Cambisol	27,697	226
Cambisol syr/thiosulfate 2nd SC	7,882	58
Cambisol syr/thiosulfate 5th SC	11,047	36
Cambisol syr/Cl-OHPA 2nd SC	5,327	34
Cambisol syr/Cl-OHPA 5th SC	18,683	38
Cambisol syr/2,4,6-TCP 2nd SC	16,547	41
Cambisol syr/2,4,6-TCP 5th SC	47,200	38
Cambisol syr 2nd SC	11,895	40
Cambisol syr 5th SC	13,361	32
Luvisol	17,144	229
Luvisol syr/thiosulfate 2nd SC	8,248	82
Luvisol syr/thiosulfate 5th SC	16,037	35
Gleysol	9,182	117
Gleysol syr/thiosulfate 3rd SC	4,538	53
Gleysol syr/thiosulfate 5th SC	60,544	46
Pelosol	32,946	373
Pelosol syr/thiosulfate 2nd SC	9,289	82
Pelosol syr/thiosulfate 5th SC	20,351	35
Podsol	16,112	87
Podsol syr/thiosulfate 2nd SC	10,647	46
Podsol syr/thiosulfate 3rd SC	16,960	32

3.6.2 Soil microbial communities

The soil microbial communities were mainly dominated by four phyla: the Acidobacteria, Actinobacteria, Bacteroidetes and Proteobacteria (see **Figure 3.16**). This stays in accordance with previous studies (Janssen, 2006). Despite these overarching dominant phyla, the species abundance and diversity were soil-specific. The podsol showed the poorest diversity at phylum level and was essentially restricted to the Acidobacteria, Actinobacteria and Proteobacteria. In contrast to this, the less acidic soils showed a higher diversity at phylum level. Since all of the sampled soils were, with a little less of extent in the pelosol, oxic soils, most microorganisms identified were aerobes or facultative anaerobes. Members of the Bacteroidetes, which is a phylum comprised of strict anaerobes, could also be identified in all soils, confirming the previously reported existence of anaerobic bacteria in aerated soils (see for example Peters & Conrad, 1995).

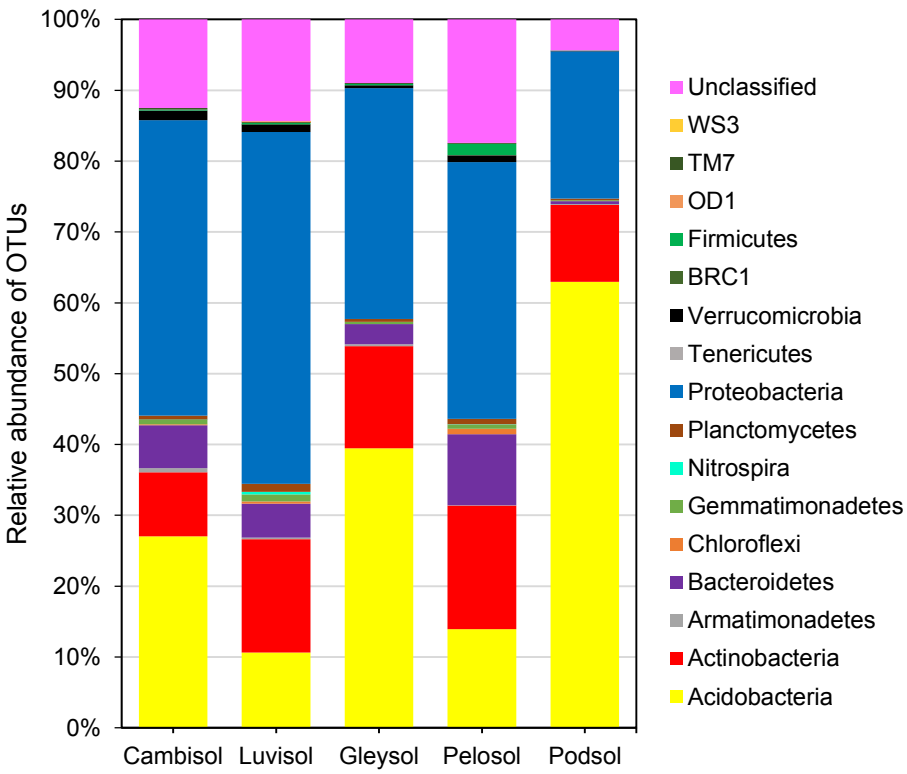


Figure 3.16: Relative abundance of phyla expressed as the percentage of total OTUs in topsoils.

The relative abundance of the Acidobacteria correlated with the pH of topsoils and was highest in the podsol (pH 2.7), while the luvisol (pH 5.9) and pelosol (pH 7.0) showed the least amount of OTUs assigned to this phylum. This stays in accordance with previously published results (Hartman et al., 2008; Jones et al., 2009). The main Acidobacteria groups that were identified in soils were the groups 1–7, 9–11, 13–18, 20 and 22. Their distribution varied depending on the soil. In contrast to the Acidobacteria, the distribution of the Actinobacteria, Bacteroidetes and Proteobacteria did not seem to be influenced by the pH. However, the Bacteroidetes could barely be detected in the most acidic soil (the podsol). As stated above, the distribution and abundance of species was dependent on the soil. Species that could be identified in most soils are summarized in **Table 3.11**. Among the Proteobacteria, the class of the Alphaproteobacteria was dominating. Most genera listed under the Alphaproteobacteria in **Table 3.11** are either known as denitrifying bacteria (Ryuda et al., 2011; Venkatramanan et al., 2013) or nitrogen-fixing symbionts (Okubo et al., 2013). Only one species belonging to the Epsilonproteobacteria was found in soils: 0.03% of total Proteobacteria OTUs were assigned to the genus *Sulfurospirillum*, a non-obligate organohalide respirer (Scholz-Muramatsu et al., 1995), in the podsol.

The phylum Firmicutes, to which *Desulfitobacterium* spp. and most acetogens belong, played a neglectable role in the soil microbial communities. Less than 1% of OTUs could be assigned to this group in all soils except for the pelosol, where they accounted for 1.5% of the community. No OTUs could be assigned to the genus *Desulfitobacterium*. A portion of the community remained unclassified at phylum level (4% of OTUs in the podsol and 9–17% in the remaining soils).

Table 3.11: Relative abundance of OTUs belonging to common genera found in most soils, expressed as the percentage of total OTUs of the corresponding phylum. Abbreviations: n.d. (not detected).

	Phylum/class	Genus	Cambisol	Luvisol	Gleysol	Pelosol	Podsol
Proteobacteria	α-Proteobacteria	*Afipia*	0.8	1.5	0.7	0.9	0.8
		Agromonas	0.1	0.6	0.1	0.2	0.6
		Bradyrhizobium	0.8	1.1	0.3	0.2	4.2
		Caulobacter	2.0	0.7	4.0	0.2	2.9
		Phenylobacterium	0.7	0.5	<0.1	0.6	0.4
	β-Proteobacteria	*Burkholderia*	8.5	0.1	4.1	n.d.	0.7
		Curvibacter	<0.1	<0.1	<0.1	<0.1	n.d.
		Janthinobacterium	<0.1	0.1	0.2	<0.1	n.d.
		Variovorax	0.3	0.3	0.2	0.3	n.d.
	δ- Proteobacteria	*Sorangium*	0.2	0.2	0.2	0.2	0.1
	γ-Proteobacteria	*Pseudomonas*	0.1	0.2	2.2	0.4	<0.1
		Rudaea	0.6	0.1	1.1	<0.1	0.1
		Steroidobacter	<0.1	0.2	0.1	0.8	0.1
	ε-Proteobacteria	*Sulfurospirillum*	n.d.	n.d.	n.d.	n.d.	<0.1
	Actinobacteria	*Conexibacter*	3.1	1.3	2.3	0.2	1.1
		Mycobacterium	1.5	0.6	3.4	0.2	1.5
		Solirubrobacter	0.5	2.6	0.2	2.6	0.1
	Bacteroidetes	*Ferruginibacter*	1.3	6.9	0.4	2.3	3.0
		Mucilaginibacter	13.6	1.5	41.8	0.1	3.0
	Firmicutes	*Bacillus*	2.2	2.6	n.d.	3.3	n.d.
		Pasteuria	39.1	76.9	n.d.	31.7	n.d.
		Sporosarcina	6.5	2.6	n.d.	0.2	n.d.

3.6.3 Community structure in enrichments with syringate/thiosulfate

The soil microbial communities were mainly dominated by aerobes or facultative anaerobes and up to some extent by strict anaerobes (Bacteroidetes, Firmicutes). Upon forcing a growth-selective process on these initial communities under anaerobic conditions, namely the *O*-demethylation of syringate coupled to the reduction of thiosulfate, a community shift favoring the strict anaerobes occured. During the intermediate sub-cultivation step on syringate/thiosulfate, the microbial communities of all soil enrichments were dominated by bacteria of the phylum Firmicutes (see **Figure 3.17**).

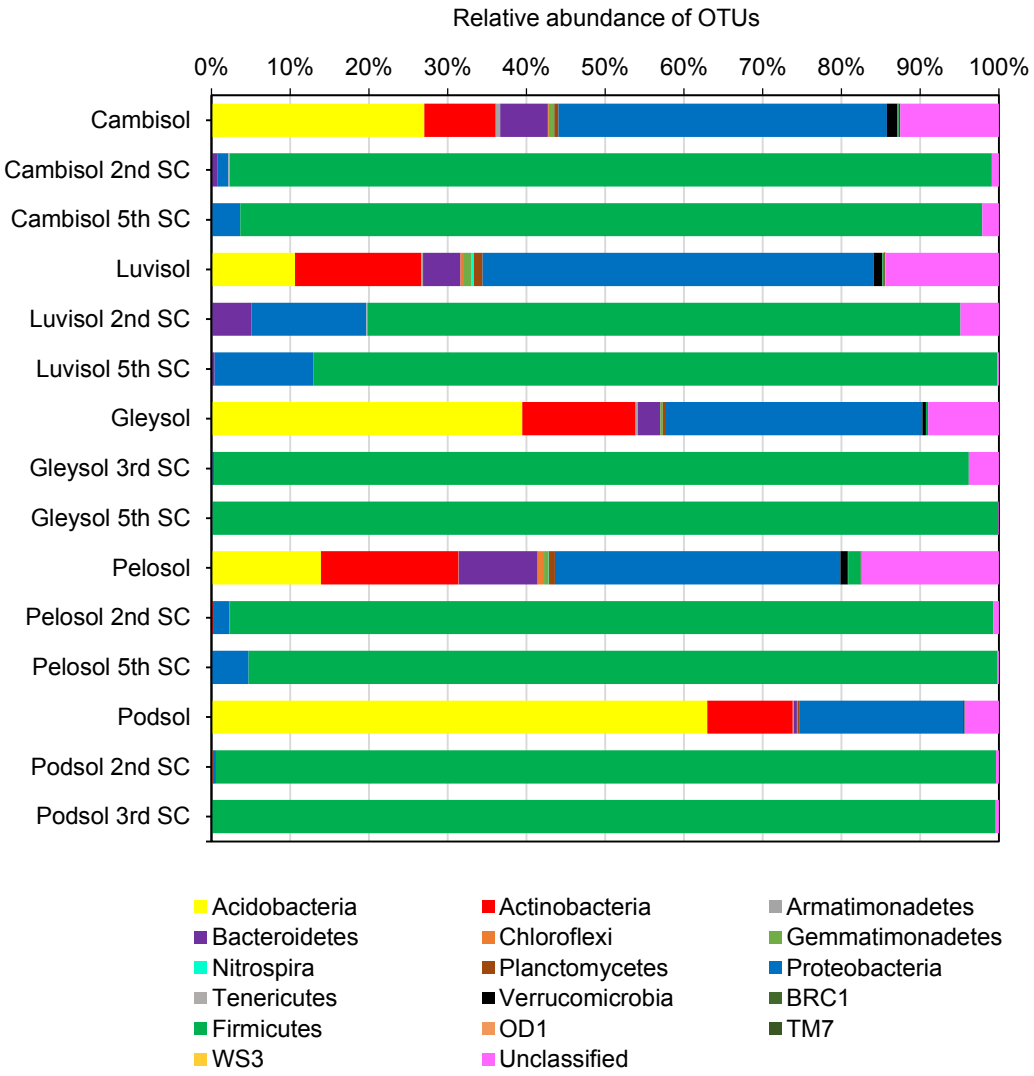


Figure 3.17: Relative abundance of phyla expressed as the percentage of total OTUs in topsoils and in enrichment cultures amended with syringate and thiosulfate. Abbreviations: SC (sub-culture).

At the end of the intermediate sub-cultivation step, the relative amount of OTUs assigned to the Firmicutes was ≥ 94% in all enrichment cultures except for the luvisol. In the latter, 75% of OTUs were assigned to this phylum. A concomitant loss of OTUs belonging to the remaining phyla was observed, although the Proteobacteria and Bacteroidetes, along with those OTUs unclassified at the phylum level, could still be detected in the enrichment cultures. During the last sub-cultivation step of each enrichment, the distribution and relative abundance of phyla was similar to the intermediate sub-cultivation. This indicates that the major community shift in response to the growth substrates ends during the early stages of enrichment, probably at the end of the first or the second sub-cultivation step. While in the cambisol, gleysol, pelosol and podsol enrichment cultures the relative amount of Firmicutes OTUs remained similar to the intermediate sub-cultivation step, a further enrichment could be observed for the luvisol culture,

where the amount of Firmicutes OTUs increased from 75% in the second to 87% in the fifth sub-cultivation step.

Desulfitobacterium sp. OTUs could be detected in all enrichments except for the podsol. In the cambisol, luvisol and pelosol enrichment cultures, the OTUs were exclusively identified during the intermediate sub-cultivation step, while in the gleysol culture OTUs could also be detected in the fifth sub-cultivation. OTUs could also be assigned to unclassified members of the family Peptococcaceae. The amount of *Desulfitobacterium* spp. OTUs and unclassified Peptococcaceae OTUs, in which unclassified desulfitobacteria might have been binned, are summarized in **Table 3.12**.

Table 3.12: Number of OTUs assigned to the genus *Desulfitobacterium* and to unclassified members of the family Peptococcaceae in each enrichment culture. The unclassified Peptococcaceae might contain uncultured or unclassified *Desulfitobacterium* species. The first value represents the absolute number of OTUs assigned to *Desulfitobacterium* spp., while the value in parenthesis represents the relative abundance in percent among all OTUs assigned to the Firmicutes. Abbreviations: n.d. (not detected).

Enrichment	Sub-cultivation	*Desulfitobacterium* spp.	Unclassified Peptococcaceae
Cambisol	2	272 (3.6)	16 (0.2)
	5	n.d.	n.d.
Luvisol	2	195 (3.2)	13 (0.2)
	5	n.d.	2 (0.01)
Gleysol	3	419 (9.6)	43 (1.0)
	5	106 (0.1)	n.d.
Pelosol	2	20 (0.2)	65 (0.7)
	5	n.d.	9 (0.05)
Podsol	2	n.d.	54 (0.5)
	3	n.d.	2 (0.01)

The absence of any *Desulfitobacterium* spp. OTUs in the podsol enrichment culture contradicts the results obtained via 16S and FTHFS qPCR detection. In order to exclude a possible primer mismatch during qPCR analyses that might have caused false positive signals in podsol enrichment cultures, a specific primer pair that exclusively targets the rdhA6 gene in *D. hafniense* DCB-2 was used according to the protocol of Mac Nelly et al. (2014). The rdhA6 gene encodes the Cl-OHPA reductive dehalogenase, and the primer pair was designed to discriminate against similar reductive dehalogenases in other *Desulfitobacterium* spp. and other organohalide respiring organisms. A 131 bp-sized fragment could be obtained in both podsol replicates via endpoint PCR (**Figure 3.18**), confirming the presence of *D. hafniense* in these cultures as determined before by cloning of qPCR products (see **Table 3.8**).

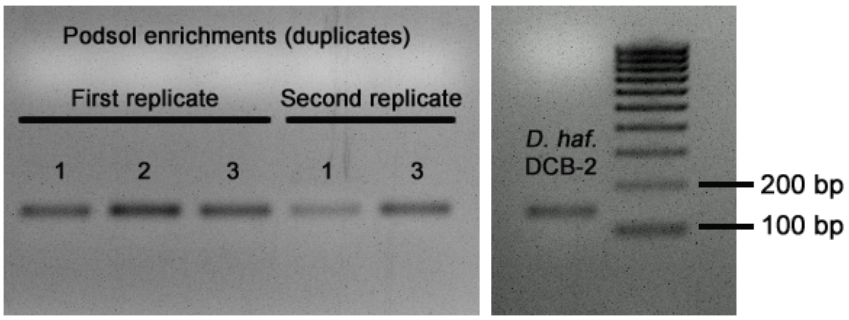


Figure 3.18: Detection of the rdhA6 gene of *D. hafniense* DCB-2 in podsol enrichment cultures. The PCR product has a size of 131 bp (Mac Nelly et al., 2014). The numbers above each band represent the corresponding sub-cultivation step on syringate/thiosulfate. DNA from the second sub-cultivation of the second podsol replicate had been exhausted in qPCR and community analysis. Thus, it could not be analyzed. Genomic DNA of *D. hafniense* DCB-2 was used as positive control and was separated on the same gel. Abbreviations: *D. haf.* (*D. hafniense*).

The absence of *Desulfitobacterium* spp. OTUs in podsol enrichments can be explained by a lower sensitivity and resolution of the community analysis. However, it cannot be excluded that novel *Desulfitobacterium* spp. might have been assigned to the group of the unclassified Peptococcaceae.

A set of six Firmicutes genera (*Sporomusa*, *Alkalibaculum*, *Sporobacterium*, *Tissierella*, *Clostridium*, *Anaerovorax*) were found to dwell in each enrichment culture, regardless of the original soil sample. They were the most abundant microorganisms in the enrichment cultures. With the exception of *Anaerovorax* and *Tissierella*, members of these genera have been described as acetogens and/or have been reported capable of cleaving the ether bond in methoxylated aromatic compounds (*Sporomusa*: Stupperich & Konle, 1993 and Kuhner et al., 1997; *Alkalibaculum*: Allen et al., 2010; *Sporobacterium*: Mechichi et al., 1999a; *Clostridium*: e.g. Mechichi et al., 1999b). Even though *Tissierella* is not described as an acetogen, its former integration into the genus *Clostridium* (Bae et al., 2004) might suggest its potential to cleave the ether bond of methoxylated aromatics. In contrast to these genera, *Anaerovorax* is mainly known for its fermentation of putrescine (tetramethylenediamine), a foul-smelling compound produced by the break-down of amino acids in corpses, to acetate, butyrate, NH_4^+ and H_2 (Matthies et al., 1989 & 2000). This suggests that the metabolic contribution of *Anaerovorax* in enrichment cultures is limited to the hydrolysis of peptides that originate from lysed bacteria. The relative abundance of OTUs assigned to the genera described above among the total Firmicutes OTUs are summarized in **Table 3.13**, and a figure depicting the composition of the Firmicutes in these enrichment cultures can be found in the APPENDIX section (**Figure 6.12**). Nevertheless, the abundance and composition of the bacterial communities were specific for each enrichment culture.

Table 3.13: Relative abundance of the most frequent Firmicutes genera identified in syringate/thiosulfate enrichments. The results are expressed as percent of total Firmicutes OTUs. Abbreviations: SC (sub-cultivation), n.d. (not detected).

Enrichment	SC	*Sporomusa*	*Alkalibaculum*	*Sporobacterium*	*Tissierella*	*Clostridium*	*Anaerovorax*
Cambisol	2	5.5	18.8	5.1	2.3	9.0	0.8
	5	19.5	50.4	4.1	2.7	1.8	0.1
Luvisol	2	0.6	2.9	0.4	8.0	5.6	1.4
	5	93.6	0.1	0.05	n.d.	1.1	0.4
Gleysol	3	0.4	23.4	0.6	2.2	4.8	0.7
	5	n.d.	74.7	0.9	0.5	7.8	0.02
Pelosol	2	n.d.	3.5	3.1	1.5	3.6	0.1
	5	n.d.	2.8	53.0	3.9	8.2	0.1
Podsol	2	0.06	n.d.	0.01	41.9	8.9	n.d.
	3	0.03	0.01	0.01	14.9	75.6	n.d.

In contrast to most acetogens, a *Sporobacterium* species has been reported capable of ring cleavage under anaerobic conditions (Mechichi et al., 1999), which might explain the non-stoichiometric recovery of gallate in enrichment cultures. Aside from the genera presented above, also *Sedimentibacter* sp. (also an acetogen) and *Desulfosporosinus* sp. (non-acetogen and closest relative of *Desulfitobacterium*) OTUs were identified in most enrichment cultures. Co-enriched Proteobacteria were almost exclusively affiliated to the family of Enterobacteriaceae (Gammaproteobacteria) and comprised genera such as *Buttiauxella*, *Citrobacter, Enterobacter* and *Serratia*. Members of the Enterobacteriaceae have been reported before to perform anaerobic *O*-demethylation of methoxylated aromatics and subsequent ring-cleavage (Grbic-Galic, 1986), further providing an explanation for the non-stoichiometric recovery of gallate in the enrichment cultures. At the end of the final sub-cultivation, the dominating Firmicutes genera were: *Alkalibaculum* (50.4%) and *Sporomusa* (19.5%) in the cambisol; *Sporomusa* (93.6%) and unclassified Veillonellaceae (2.1%) in the luvisol; *Alkalibaculum* (74.7%) and unclassified Lachnospiraceae (8.6%) in the gleysol; *Sporobacterium* (53.0%) and unclassified Lachnospiraceae (17.8%) in the pelosol and *Clostridium* (75.6%) and *Tissierella* (14.9%) in the podsol enrichment culture.

3.6.4 Community structure in cambisol enrichments with alternate electron acceptors

The enrichment of *Desulfitobacterium* spp. was also studied in cambisol enrichment cultures by substituting thiosulfate for chlorophenolic compounds, namely Cl-OHPA and 2,4,6-TCP. A control enrichment that lacked an alternate acceptor was also studied. *Desulfitobacterium* 16S rRNA gene copies could be detected in every culture by qPCR, although the enriched amount was not as high as in enrichments with thiosulfate (see 3.4.2). The communities were analyzed as described for the enrichments with syringate/thiosulfate, also using the sub-cultivation step with maximum relative amount of *Desulfitobacterium* gene copies (in this case the second sub-cultivation step) and the last sub-cultivation step. The corresponding communities are displayed in **Figure 3.19**.

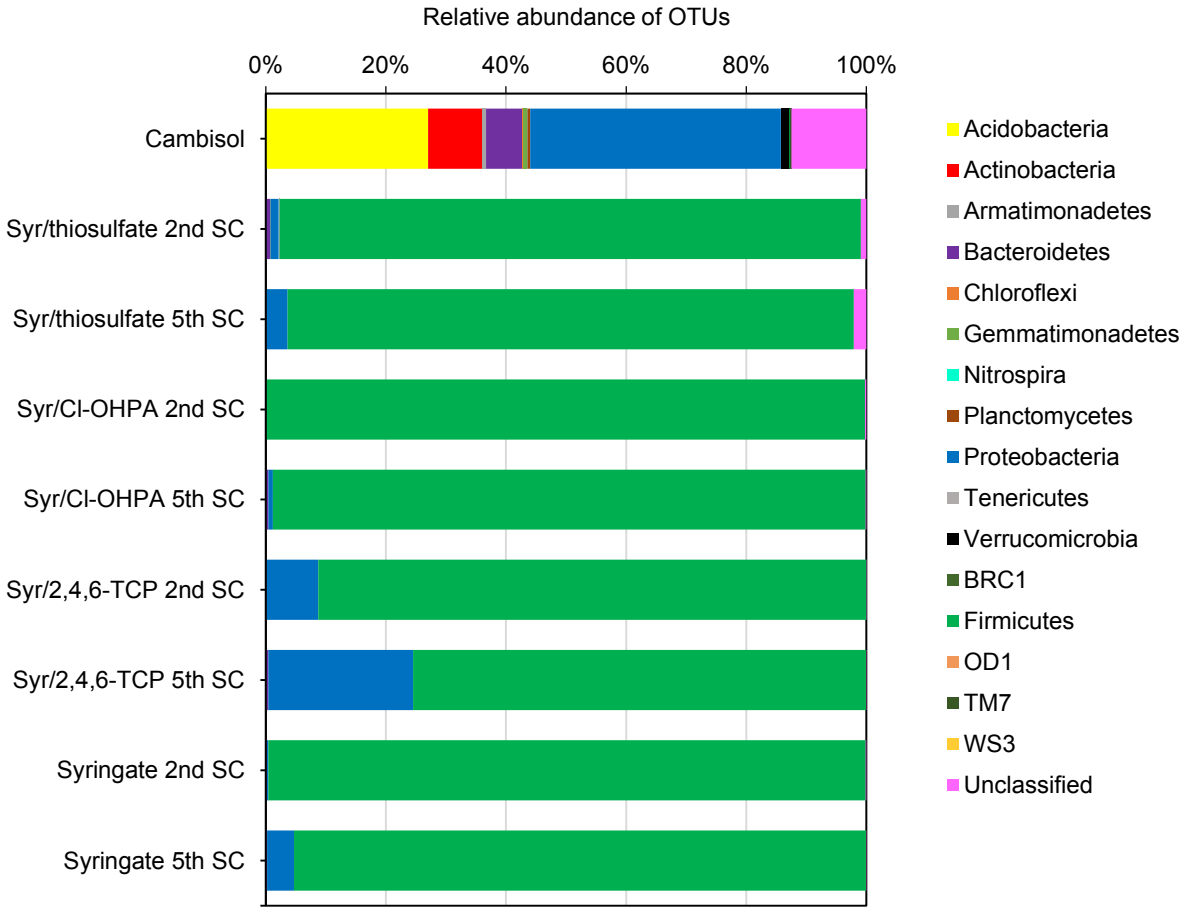


Figure 3.19: Relative abundance of phyla expressed as the percentage of total OTUs in cambisol topsoil and cambisol enrichment cultures. Abbreviations: SC (sub-cultivation), Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), TCP (trichlorophenol).

The overall community structure of these additional cambisol enrichments resembled the structure of the enrichment with syringate/thiosulfate. The results demonstrate that the community structure at phylum level is influenced by the electron donor (syringate), which is a highly selective substrate, while the electron acceptor does not seem to significantly influence the overall distribution of phyla. This is probably due to the fact that the dissolved HCO_3^- in the growth medium will automatically lead to the enrichment of acetogenic Firmicutes from environmental samples in combination with a phenyl methyl ether as electron donor. Among the Firmicutes, the genera listed before in **Table 3.13** were also found to account for the highest portion of Firmicutes OTUs. *Sporomusa* OTUs made up at least 76% of Firmicutes OTUs during the fifth sub-cultivation step on each enrichment culture amended with syringate and a chlorophenol as electron acceptor, as well as in the control enrichment culture. A figure depicting the composition of the Firmicutes in cambisol enrichment cultures with different electron acceptors can be found in the APPENDIX section (**Figure 6.13**). OTUs assigned to the genus *Desulfitobacterium* could not be identified in any of the chlorophenol-amended cultures nor in the control enrichment, which contradicts the 16S rRNA and FTHFS qPCR assays, as described before. To rule out the possibility of a false positive signal during qPCR analyses, and to verify the presence of any *Desulfitobacterium* species in these enrichment cultures, the rdhA6 gene of *D. hafniense* DCB-2 was used again as a marker gene to verify the presence of *D. hafniense* as described in 3.6.3. The rdhA6 gene could be detected in all three enrichment cultures, although it it remained undetectable in some sub-cultivations (**Figure 3.20**). The results prove the presence of *D. hafniense* in the enrichment cultures even though no *Desulfitobacterium* OTUs could be detected via community analysis. The contribution of other *Desulfitobacterium* spp. to the dehalogenation of Cl-OHPA and 2,4,6-TCP cannot be excluded, as the utilization of these compounds is not an ability restricted to *D. hafniense* (see Villemur et al., 2006) and the presented PCR assay discriminates against non-*hafniense* species. As described above, these results, in combination with the 16S and FTHFS qPCR results, point to a lower sensitivity of the community analysis as compared to the other methods applied.

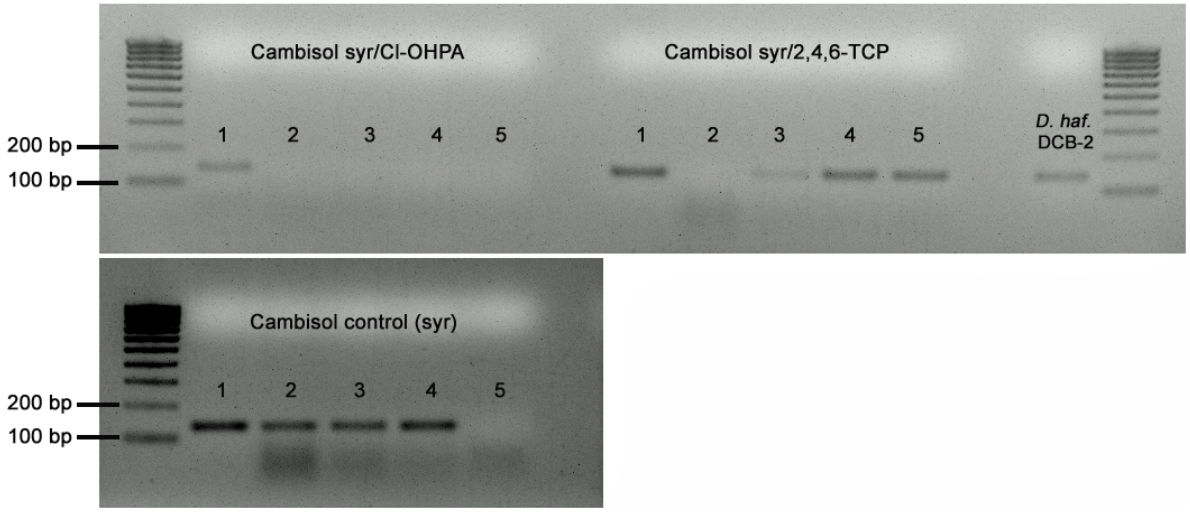


Figure 3.20: Detection of the rdhA6 gene of *D. hafniense* DCB-2 in cambisol enrichment cultures. The PCR product has a size of 131 bp. The numbers above each band represent the corresponding sub-cultivation step. Abbreviations: Syr (syringate), Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), 2,4,6-TCP (2,4,6-trichlorophenol), *D. haf.* (*D. hafniense*).

In enrichments amended with 2,4,6-TCP, a significantly higher amount of Proteobacteria could be identified compared to enrichment cultures amended with syringate/thiosulfate. They belonged, as described before for the enrichment cultures with syringate/thiosulfate, almost exclusively to the Gammaproteobacteria and mostly belonged to the Enterobacteriaceae. This is probably due to the ability of some Enterobacteriaceae to demethylate methoxylated aromatic compounds (Grbic-Galic, 1986). However, OTUs were also assigned to genera that were not present in enrichments in which thiosulfate was the terminal electron acceptor. These OTUs were assigned to genera of the Enterobacteriaceae that have been reported capable of reductive dechlorination of organohalide compounds under aerobic or anaerobic conditions: *Enterobacter* (Sharma & McCarty, 1996), *Shigella* (Karn & Balda, 2013) and *Serratia* (Singh et al., 2007), with 0.02%, 58.6% and 0.02% of total Proteobacteria OTUs, respectively. Nevertheless, the Proteobacteria played a minor role compared to the Firmicutes. **Table 3.14** presents the dominating Firmicutes species at the end of the final sub-cultivation for each of the additional cambisol enrichments.

Table 3.14: Dominating Firmicutes genera at the end of the final sub-cultivation step in cambisol enrichment cultures amended with chlorophenols and in the control enrichment. Abbreviations: Syr (syringate), Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), TCP (trichlorophenol).

Enrichment	Dominating Firmicutes genera	Relative abundance among Firmicutes OTUs (%)
Syr/Cl-OHPA	*Sporomusa*	75.8
	Clostridium	11.3
Syr/2,4,6-TCP	*Sporomusa*	85.4
	Uncl. Veillonellaceae	11.1
Control (syr)	*Sporomusa*	85.2
	Sporobacterium	8.7

3.6.5 Alpha diversity of soil and enriched communities

The term alpha diversity (α diversity) was first proposed by Robert Whittaker and describes the species richness in a sample. The abundance of each species is not taken into account, but only its presence, and thus those species that dominate a habitat give as much weight to the alpha diversity as rare species within the same habitat (Whittaker, 1972).

Analysis of alpha diversity by rarefaction

The species richness in each of the analyzed communities was assessed by rarefaction. Rarefaction curves (also known as collector's curves or species accumulation curves) consist of a plot of the number of observed species (or, in this case, OTUs) as a function of the number of samples taken (or, in this case, number of reads or sequences). Therefore, rarefaction is a powerful tool to assess if enough sampling effort has been done. Rarefaction curves were calculated with mothur for all soil microbial communities and their corresponding enrichments by taking a sub-sample of 4,538 random reads. This amount corresponds to the lowest amount of reads obtained for one of the samples. The rarefaction curves are displayed in **Figure 3.21 A–H**.

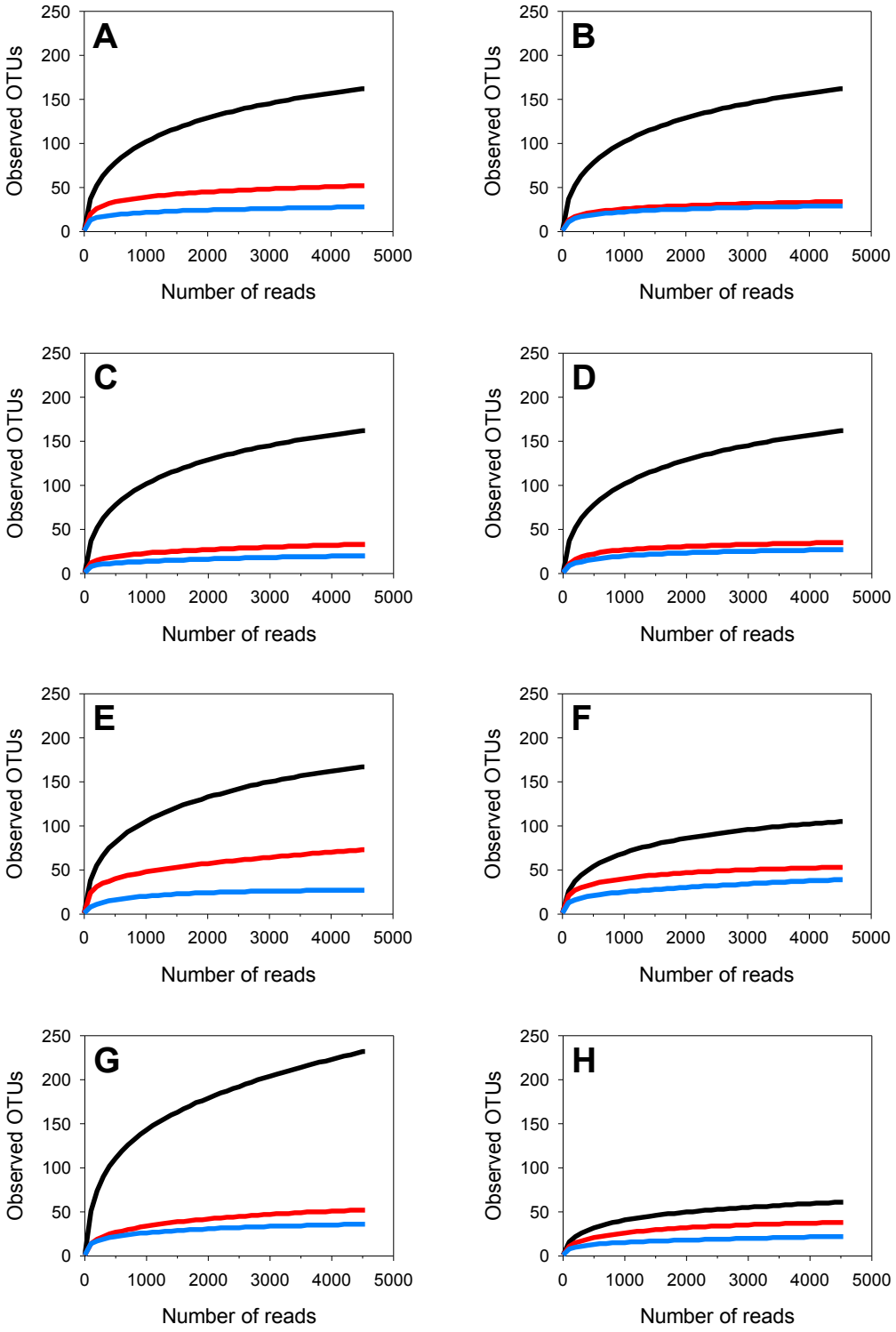


Figure 3.21: Rarefaction curves of soil and enriched microbial communities. A sub-sample of approximately 4,500 reads was selected for the following series: cambisol with syringate/thiosulfate (A), cambisol with syringate/Cl-OHPA (B), cambisol with syringate/2,4,6-TCP (C), cambisol with syringate (D), luvisol with syringate/thiosulfate (E), gleysol with syringate/thiosulfate (F), pelosol with syringate/thiosulfate (G) and podsol with syringate/thiosulfate (H). In each panel, the initial soil microbial community (black curve) and the microbial community of the 2nd (red curve; for gleysol, third SC) and 5th SC (blue curve; for podsol, third SC) is shown. Abbreviations: Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), OTU (operational taxonomic unit), SC (sub-culture), TCP (trichlorophenol).

All rarefaction curves, especially those depicting the species richness of the soil microbial communities (**Figure 3.21**, green curves), started as a steep curve, since the OTUs located at the beginning of the curve belong to the abundant species. With increasing sampling effort, the number of additional OTUs obtained decreased in all curves, as only the OTUs belonging to less abundant species were added to the plot. With the exception of the podsol series (**Figure 3.21 H**), the calculated rarefaction curves demonstrated in all cases that the species diversity was significantly higher in soil microbial communities and that it was narrowed by selective enrichment. Within these subsamples, the number of observed species in soils decreased until the end of the enrichment process by an approximate factor of 3 in the gleysol and podsol, 5 in the cambisol and 6 in the luvisol and pelosol. Among the soils, the pelosol (**Figure 3.21 G**) showed the highest species richness, followed by the cambisol (**Figure 3.21 A-D**) and the luvisol (**Figure 3.21 E**), which had an almost equal number of observed species. The gleysol (**Figure 3.21 F**) and especially the podsol (**Figure 3.21 H**) showed the lowest species richness. The species richness of topsoils correlated with the measured pH (**Table 3.8**), with the less acidic soils having a higher species diversity than the more acidic ones. The number of OTUs observed per sub-sample and the species coverage of the corresponding sub-samples are listed in **Table 3.15**.

A species coverage of ≥ 98% could be obtained for all sub-samples, including the soil microbial communities. The shape of the rarefaction curves indicates that for capturing the diversity in enrichment cultures, an even smaller sub-samples (less reads) would have sufficed, e.g. 1000 reads. A higher number of reads is needed for soil microbial communities, though, given the higher species diversity. Roughly 4,500 reads probably represent the optimal value for soils given the species coverage in the sub-samples. For the podsol microbial community, 1000 reads would probably have sufficed for a nearly complete species coverage given its low diversity.

Table 3.15: OTU coverage and number of observed OTUs in a sub-sample consisting in 4538 reads of each soil and enrichment community. Abbreviations: syr (syringate), Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), TCP (trichlorophenol), SC (sub-cultivation).

Group	Species coverage (%)	Species observed
Cambisol	99.14	162
Cambisol syr/thiosulfate 2nd SC	99.78	52
Cambisol syr/thiosulfate 5th SC	99.89	28
Cambisol syr/Cl-OHPA 2nd SC	99.87	34
Cambisol syr/Cl-OHPA 5th SC	99.91	29
Cambisol syr/2,4,6-TCP 2nd SC	99.82	33
Cambisol syr/2,4,6-TCP 5th SC	99.91	20
Cambisol syr 2nd SC	99.89	35
Cambisol syr 5th SC	99.91	27
Luvisol	99.14	167
Luvisol syr/thiosulfate 2nd SC	99.49	73
Luvisol syr/thiosulfate 5th SC	99.96	27
Gleysol	99.51	105
Gleysol syr/thiosulfate 3rd SC	99.85	53
Gleysol syr/thiosulfate 5th SC	99.71	39
Pelosol	98.41	232
Pelosol syr/thiosulfate 2nd SC	99.78	52
Pelosol syr/thiosulfate 5th SC	99.85	36
Podsol	99.69	61
Podsol syr/thiosulfate 2nd SC	99.85	38
Podsol syr/thiosulfate 3rd SC	99.89	22

Analysis of alpha diversity by Simpson's reciprocal index

Simpson's reciprocal index (also known as inverse Simpson index) was used as a second estimate for the alpha diversity in samples. **Table 3.16** presents the values for D' as calculated by mothur. Simpson's diversity index (D) describes the probability of picking the same OTU twice if two random OTUs are picked from a sample. If no diversity exists in a sample (e.g. only one species is present), D will equal 100% (or 1). The ideal diversity of 0% describes the hypothetical case of infinite diversity, in which there is no chance of picking the same species randomly twice because each species is represented by only one individual. Simpson's reciprocal index (D') starts with 1 as the lowest value (in this case, D = 100% or 1). The higher D', the higher the diversity in a sample. The maximum D' value equals the number of species in the sample.

Table 3.16: Simpson's index (D) and Simpson's reciprocal index (D') for soil and enriched microbial communities. Abbreviations: syr (syringate), Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), TCP (trichlorophenol), SC (sub-cultivation).

Group	D	D'
Cambisol	0.06	16.6
Cambisol syr/thiosulfate 2nd SC	0.14	7.0
Cambisol syr/thiosulfate 5th SC	0.27	3.7
Cambisol syr/Cl-OHPA 2nd SC	0.36	2.8
Cambisol syr/Cl-OHPA 5th SC	0.51	1.9
Cambisol syr/2,4,6-TCP 2nd SC	0.36	2.4
Cambisol syr/2,4,6-TCP 5th SC	0.63	2.6
Cambisol syr 2nd SC	0.41	2.7
Cambisol syr 5th SC	0.39	1.6
Luvisol	0.06	17.3
Luvisol syr/thiosulfate 2nd SC	0.08	13.2
Luvisol syr/thiosulfate 5th SC	0.62	1.6
Gleysol	0.16	6.3
Gleysol syr/thiosulfate 3rd SC	0.10	9.6
Gleysol syr/thiosulfate 5th SC	0.40	2.5
Pelosol	0.03	31.3
Pelosol syr/thiosulfate 2nd SC	0.44	2.3
Pelosol syr/thiosulfate 5th SC	0.27	3.6
Podsol	0.36	2.8
Podsol syr/thiosulfate 2nd SC	0.23	4.3
Podsol syr/thiosulfate 3rd SC	0.54	1.9

Typically, the D' value is higher for soils than for the corresponding enrichments, indicating a broader species richness in the soils than in the enrichment cultures. This means that the soil microbial communities relate closer to an ideal, infinite diversity in which the same OTU cannot be picked twice from a sample when picking two random OTUs, according to the definition of Simpson's diversity index. Moreover, in most cases D' is smaller in the last sub-cultivation step of each enrichment compared to the intermediate sub-cultivation step. This means that even though the distribution of phyla does not significantly change from the intermediate to the last sub-cultivation step of each enrichment, a set of microorganisms is out-competed during the late steps of the enrichment process. The results obtained from rarefying the data could be verified by Simpson's reciprocal index

3.6.6 Beta diversity of soil and enriched communities

The communities of soils and enrichments were further compared based on the evenness of species distribution and abundance (beta diversity). For this, the dissimilarity indices of Jaccard and Yue & Clayton were calculated with mothur (see 2.5.3). Jaccard's index describes the similarity in community membership between two samples. For this, the ratio between shared OTUs and total amount of OTUs in two samples is calculated. The pair-wise comparison of Jaccard index values (**Figure 3.22**) showed a rather high similarity in community membership of soil microbial communities when compared to one another. An even higher similarity was true for the enriched communities. This is feasible, as it was shown above that a certain set of acetogens is present in almost every enrichment culture, and thus a high number of shared OTUs can be expected when comparing the communities of enrichment cultures. However, when comparing the initial soil microbial communities to the enriched communities, low Jaccard indices were obtained, proving a significant shift of the community upon enriching and the loss of genera belonging to the out-competed phyla and less shared OTUs. The results obtained for the Yue & Clayton dissimilarity index differed from the ones obtained for Jaccard's dissimilarity (**Figure 3.23**). In contrast to Jaccard's index, the Yue & Clayton index describes the similarity in community structure between two samples. For this, the relative abundance of OTUs is taken into account. The pair-wise comparison of Yue & Clayton indices indicates that, even though a high number of shared OTUs might exist when comparing enrichment culture samples and soil samples among each other via Jaccard's index, the abundance of the shared OTUs differs. The structure of most communities appears to be unique, hence the vast majority of calculated Yue & Clayton indices is low. Only a few communities seem to share the same OTUs in similar abundancies. These are mostly cambisol enrichment cultures compared with each other, as well as to some extend the luvisol enrichment cultures compared with cambisol enrichment cultures, and the soils compared to each other.

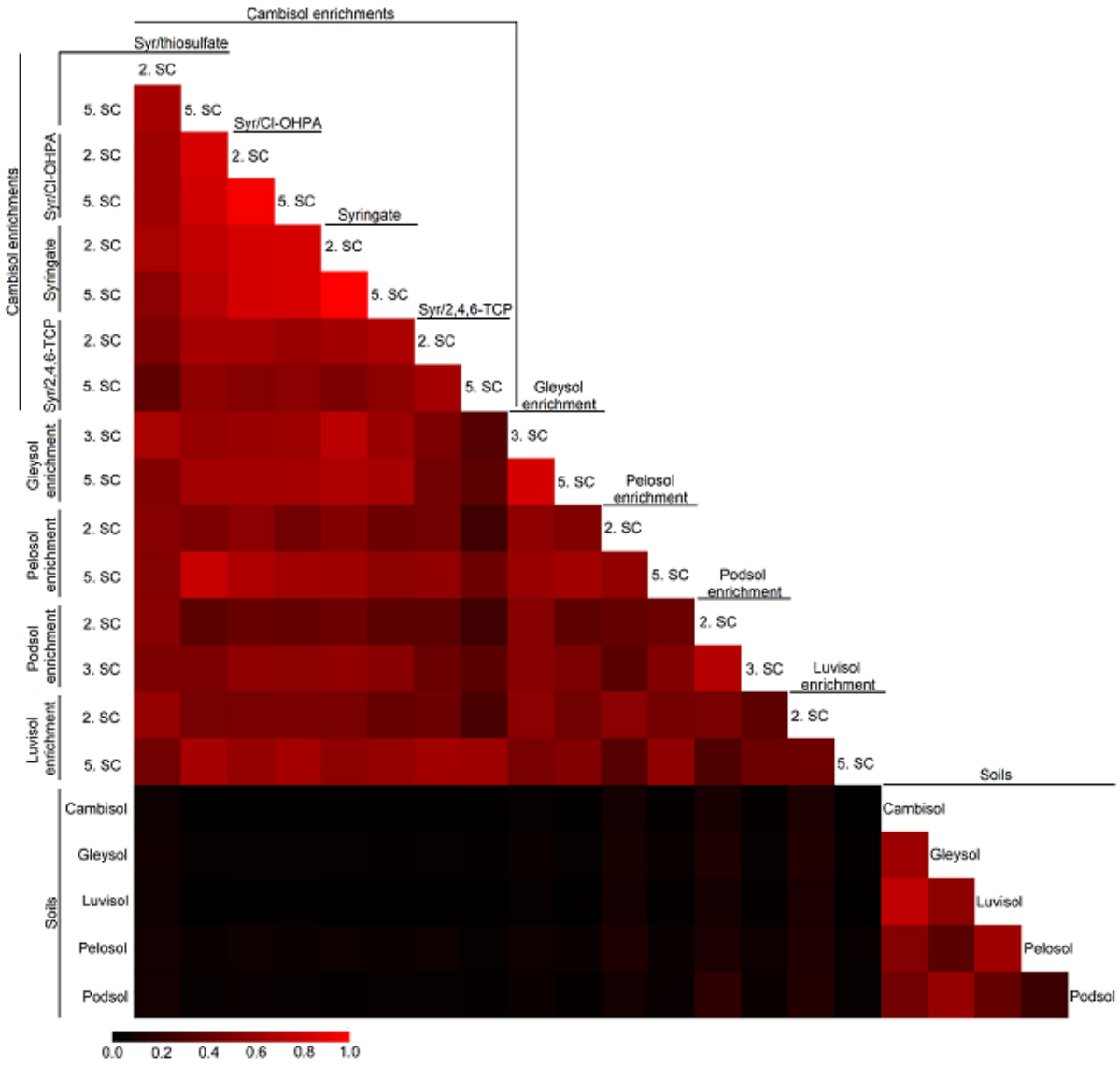


Figure 3.22: Pair-wise comparison of microbial communities from soils and enrichments by the Jaccard index of dissimilarity. A black color equals a Jaccard index of 0 (dissimilar communities), while a bright red color equals a Jaccard index of 1 (identical communities). Abbreviations: Syr (syringate), Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), TCP (trichlorophenol), SC (sub-cultivation).

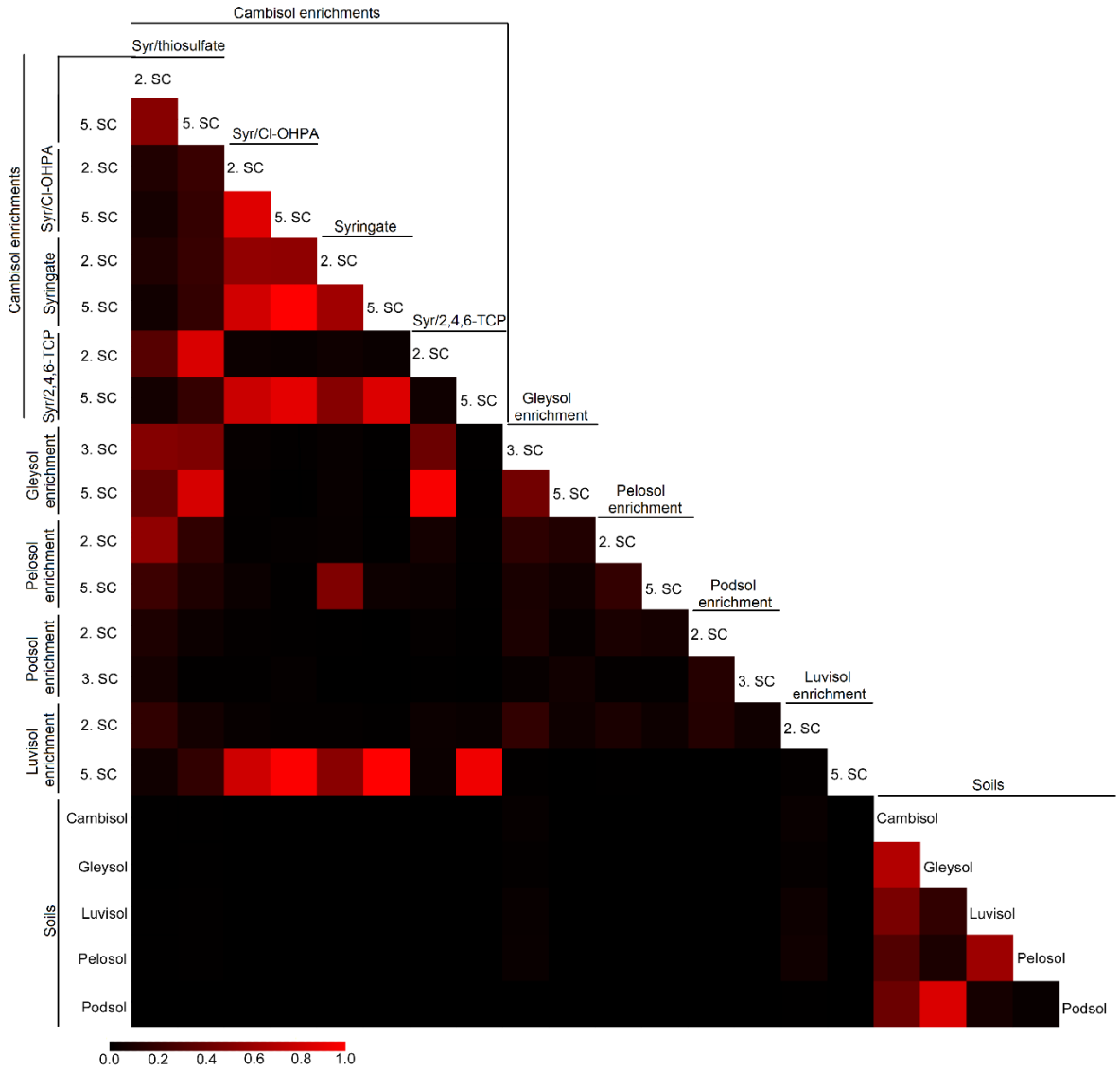


Figure 3.23: Pair-wise comparison of microbial communities from soils and enrichments by the Yue & Clayton index of dissimilarity. A black color equals a Yue & Clayton index of 0 (dissimilar communities), while a bright red color equals a Yue & Clayton index of 1 (identical communities). Abbreviations: Syr (syringate), Cl-OHPA (3-chloro-4-hydroxyphenylacetic acid), TCP (trichlorophenol), SC (sub-cultivation).

4 DISCUSSION

4.1 *O*-demethylation of phenyl methyl ethers by *Desulfitobacterium* spp.

The *O*-demethylation of vanillate, coupled to the reduction of fumarate or Cl-OHPA, was described previously for *D. hafniense* strains DCB-2 and PCE-S (Neumann et al., 2004). Nevertheless, it was not known whether *O*-demethylation might be a metabolic ability restricted to some species or a common feature among members of the genus *Desulfitobacterium*. To address this question, various *Desulfitobacterium* species were tested for *O*-demethylation of selected phenyl methyl ethers with varying electron acceptors. It could be shown that *O*-demethylation is a common feature among the members of the genus *Desulfitobacterium*, with the exception of *D. metallireducens*, a species that lacks putative demethylases in its genome. Syringate, vanillate and isovanillate have been reported to be among the most abundant phenyl methyl ethers that result from lignin degradation (Chen et al., 1982; Chen et al., 1983; Kögel, 1986) and were previously detected in soils (Whitehead, 1964; Shindo et al., 1977; Baziramakenga et al., 1995). This might explain the fact that these compounds could be demethylated by *D. chlororespirans*, *D. dehalogenans* and several *D. hafniense* strains tested in this study. The ability to convert these abundant substrates points to their use as carbon and/or energy sources by *Desulfitobacterium* spp. in terrestrial ecosystems, hinting at their involvement in the network of lignin degradation.

No clear substrate preference was observed for any of the phenyl methyl ethers tested. In contrast to syringate, vanillate and isovanillate, the *O*-demethylation of 4-hydroxyanisole was an ability exclusive to *D. chlororespirans* and *D. hafniense* strains DCB-2, DP7, PCP-1 and TCP-A. Information regarding this compound in the environment is scarce and its presence in soil has not been reported. The lack of membrane transporters for uptake in the remaining desulfitobacteria might be the logical consequence of the uncommonness of 4-hydroxyanisole in the environment. In fact, some substrates that are cleaved by purified *O*-demethylases *in vitro* cannot be used as a carbon and/or energy source *in vivo* probably for the same reason. A purified *O*-demethylase from *D. hafniense* DCB-2 exhibited the highest ether-cleaving activity towards the phenyl methyl ether guaiacol (Studenik et al., 2012), but this substrate did not serve as a growth substrate in growth experiments, pointing to the lack of membrane transporters needed for the uptake of this compound (data not shown).

Only a small set of phenyl methyl ethers was tested in this study. It cannot be excluded that additional compounds belonging to this class (e.g. ferulate, cinnamate, sinnapate…) play a role for methylotrophic metabolism in *Desulfitobacterium* spp. This question may be addressed within the framework of further growth experiments.

The *O*-demethylation of the tested phenyl methyl ethers could be coupled to the reduction of any of the provided electron acceptors except for CO_2, which is in accordance with previously published results (Neumann et al., 2004; Kreher et al., 2008). Fumarate promoted the fastest growth in combination with each phenyl methyl ether in most species and strains, but a physiological importance of this electron acceptor in the environment is unlikely. Although fumaric acid can be extracted from various soil types such as coastal soils, podsols, chernozems and luvisols, its presence is mostly restricted to particulate organic matter such as humic substances and to the rhizosphere (Grierson, 1992; Szmigielska et al., 1996; van Hees et al., 1999). In the rhizosphere, where fumarate can occur, the acidic pH and oxic conditions probably disfavor growth of desulfitobacteria. Offside the rhizosphere, the availability of fumarate in soils is probably limited, but not excluded. It is more likely that *O*-demethylation is coupled to the reduction of abundant inorganic compounds that result from the weathering process of (clay) minerals and oxides or from the mineralization of organic N- and S-containing compounds. Therefore, nitrate, thiosulfate and Fe(III) were additionally tested, as several candidate genes putatively encoding proteins involved in the reduction of these compounds had already been identified in the genomes of *Desulfitobacterium* spp. before (Nonaka et al., 2006; Kim et al., 2012). Nitrate, which in soils mainly originates from N-containing organic compounds (peptides, phospholipid fatty acids) or from the oxidation of NH_4^+ (Blume, 2010) is one of the most abundant electron acceptors in soils. Its presence in forest soils (e.g. Vitousek et al., 1982; Gebauer et al., 1988) is widely acknowledged and it might be the predominant electron acceptor whose reduction is coupled to *O*-demethylation by desulfitobacteria. In contrast to nitrate, Fe(III) mainly originates from soil mineral matter (e.g. pyrite, chalcopyrite and siderite) under weathering conditions. In soils, free Fe(III) is mostly unavailable to microorganisms, given the fact that Central European soils have a mean pH of 5.0–6.5 (Blum, 2007) and that at a pH > 2.5 Fe(III) precipitates forming oxides such as goethite (FeOOH) or haematite (Fe_2O_3) under oxic conditions (Colombo et al., 2014). However, superficial Fe(III) can be released from the mineral crystal by complexation using siderophores. Genes presumably encoding siderophore biosynthesis have been identified in the genome of *D. hafniense* Y51 (Nonaka et al., 2006). The resulting complex can be taken up by bacteria via membrane transporters, thus making the acquisition of Fe(III) possible. The presence of

thiosulfate is reported in most soils except in very humid regions (Starkey, 1950). It originates from the cycling of sulfur, an element that is contained in and released from soil mineral matter under weathering conditions (e.g. marcasite, galena and wurtzite), and to a lesser extent from mineralization of S-containing organic matter (e.g. methionine and cysteine). Given the presence of any of these compounds in soils, their utilization as terminal electron acceptors for *O*-demethylation is feasible. Compared to *O*-demethylation coupled to the reduction of fumarate, though, the demethylation rates and growth yields with nitrate, thiosulfate or Fe(III) as electron acceptors were slightly lower.

As stated above, *Desulfitobacterium* spp. could not grow by coupling the *O*-demethylation of phenyl methyl ethers to the reduction of CO_2 to acetate, although all genes needed for the conversion of CO_2 to acetate are present in the genomes of *D. hafniense* strains DCB-2 and Y51 (Kim et al., 2012; Nonaka et al., 2006, respectively) and in the genomes of the remaining *Desulfitobacterium* spp. tested in this study, including the non-demethylating *D. metallireducens*, as revealed by searches and comparisons within the KEGG database (Kanehisa & Goto, 2000; Kanehisa et al., 2014). Moreover, the activities of all enzymes required for this pathway were detected in crude extracts of *D. hafniense* strains DCB-2 and PCE-S (Kreher et al., 2008). The conversion of acetyl-CoA to acetate might be limiting under the experimental conditions chosen. This conversion is a two-steps reaction catalyzed by phosphate acetyltransferase and acetate kinase. In the first step, the acetyl group of acetyl-CoA is transferred to a phosphate group by phosphate acetyltransferase. Subsequently, acetate kinase mediates the phosphorylation of ADP to ATP with acetyl phosphate as phosphoryl donor. The disability to perform these two steps was attributed, in the case of *D. hafniense* DCB-2 and Y51, to a missing phosphate acetyltransferase (Kim et al., 2012). However, specific activities belonging to phosphate acetyltransferase and acetate kinase could be measured in the crude extracts of *D. hafniense* strains DCB-2 and PCE-S in another study (Kreher et al., 2008). The activities were low, leading to the assumption that these reactions may not be performed under *in vivo* conditions, making the presence of an alternate electron acceptor mandatory for growth (Kreher et al., 2008).

4.2 Genetic background of *O*-demethylation in *Desulfitobacterium* spp.

With the exception of *D. metallireducens*, putative demethylase systems could be identified in the genome of every *Desulfitobacterium* species analyzed in this study. No discrimination between *O*-, *N*-, *S*- or *Cl*-demethylases was possible. The highest amounts of demethylase

systems were generally found in strains of *D. hafniense*, followed by *Desulfitobacterium* sp. LBE and PCE1. *D. dehalogenans* and *D. dichloroeliminans* had the least amount of predicted demethylases. The presence of multiple demethylase operons was also described before for *D. hafniense* DCB-2 (Studenik et al., 2012) and some acetogenic bacteria as *Moorella thermoacetica* (Pierce et al., 2008).

Previous studies have shown that *O*-demethylase gene expression is induced by the corresponding substrates (Engelmann et al., 2001; Peng et al., 2011). Considering that a variety of phenyl methyl ethers can be released into environmental compartments upon lignin degradation, the presence of several *O*-demethylases in the genomes of *Desulfitobacterium* spp. represents an advantage of these organisms in such environments. However, it was shown that the methyltransferase I (MT I), which is responsible for the substrate specificity of *O*-demethylases (Engelmann et al., 2001; Schilhabel et al., 2009; Kreher et al., 2010), can have multiple substrate specificities in both *A. dehalogenans* (Kaufmann et al., 1998) and *D. hafniense* DCB-2 (Studenik et al., 2012). Some *O*-demethylases have even been shown to have an overlapping substrate specificity, as it was reported for the vanillate and veratrole *O*-demethylase of *A. dehalogenans* (Engelmann et al., 2001; Schilhabel et al., 2009). This raises the question for the need of several putative *O*-demethylases in the genomes of *Desulfitobacterium* spp., as the demethylation of several phenyl methyl ethers could be covered by only a few MT I. The same thought applies to the corrinoid protein (CP) and methyltransferase II (MT II). As these latter components function as an intermediary methyl group carrier and transferrer, respectively, only one gene copy of each would suffice. Thus, the high number of putative demethylase systems might represent paralogs of an original operon that was replicated via gene duplication and mutated in response to environmental conditions. This trait is especially pronounced in the genomes of the analyzed *D. hafniense* strains, as their genomes harbor the highest number of putative demethylase systems within the genus *Desulfitobacterium*.

The last component of *O*-demethylases, the activating enzyme (AE), is comprised within the COG3894 protein superfamily. Genes encoding for proteins belonging to this overarching class of uncharacterized metal-binding proteins are often found in the vicinity of a putative demethylase operon. It was demonstrated before that only one AE acts in the reduction of physiologically inactive [Co^{II}]-CP to active [Co^{I}]-CP in *D. hafniense* DCB-2 (Studenik et al., 2012). Nonetheless, several genes encoding for COG3894 proteins were identified in the genomes of most *Desulfitobacterium* spp., indicating that the corresponding gene products

might be involved in the reductive activation of corrinoid cofactors belonging to e.g. the corrinoid iron/sulfur protein of the Wood-Ljungdahl pathway, or to the corrinoid proteins of *N*-, *S*-, or *Cl*-demethylases.

4.3 Abundance of *Desulfitobacterium* spp. in forest and grassland topsoils

Due to the description of *Desulfitobacterium* spp. as reductively dehalogenating bacteria, common enrichment and isolation strategies have so far included the use of organohalide compounds as terminal electron acceptors (Utkin et al., 1994; Bouchard et al., 1996; Breitenstein et al., 2001). Therefore, up to date, most studies report on the presence of *Desulfitobacterium* spp. in environments contaminated with chlorinated compounds or on their enrichment from the corresponding environmental samples (e.g. Drzyzga et al., 2001; Yoshida et al., 2007). It was shown before that organohalide respirers such as *Dehalococcoides* spp. can be detected in uncontaminated forest soil (Krzmarzick et al. 2012). Obligate organohalide respirers such as *Dehalococcoides* spp. are niche specialists due to their restricted metabolism (Hug et al., 2013), hence, their presence in uncontaminated soils is unexpected and points to a natural niche in this environment for reductively dechlorinating bacteria. Similarly, a novel dechlorinating Firmicutes group has been identified in uncontaminated forest soils (Krzmarzick et al., 2014).

The presence of *Desulfitobacterium* spp. in habitats not contaminated with anthropogenic organohalide compounds has scarcely been documented. Some studies report the presence of this genus in forest soil (Lanthier et al., 2001; Hartmann et al., 2014), sediment cores of lakes (Krzmarzick et al., 2013) or heavy metal-afflicted lake and riverbed sediments (Niggemyer et al., 2001; Sánchez-Andrea et al., 2011 & 2012), but it was mostly associated with the cycling of sulfur or metals. In this study it was demonstrated that *Desulfitobacterium* spp. can be detected in soils of coniferous forest, deciduous forest and even in grassland soils. The metabolism of *Desulfitobacterium* spp. appears to be versatile enough to bypass adverse conditions such as an acidic pH or oligotrophy for survival. This assumption is supported by the detection of this genus in acidic soils that were sampled beneath coniferous forest. Since all sampling sites in this study had no known record of historical contamination, it can be assumed that, aside from the occasional dechlorination of chlorinated aryl metabolites that are produced by basidiomycetes (Verhagen et al., 1996), the presence of *Desulfitobacterium* spp. can be explained by an “alternative lifestyle”, which focuses on the use of abundant carbon and energy sources available at the corresponding sampling sites. Phenyl methyl ethers, which can be

detected in forest soils (Whitehead et al., 1964; Baziramakenga et al., 1995), might represent such abundant carbon sources in forest soils. Even though it is known that differences in the quantity and structural composition of lignin derived from softwood and hardwood species exist (Prescott et al., 2004; Rodríguez-Couto & Sanromán, 2005), structural units such as vanillate and syringate are common to both wood-type species (Hedges & Mann, 1979; Kögel, 1986) and therefore available in coniferous and deciduous forest (and grassland) soils. These compounds might therefore enable a methylotrophic lifestyle of *Desulfitobacterium* spp. in forest soils, which can, but must not necessarily, be coupled to the reductive dehalogenation of natural organochlorines. In fact, phenyl methyl ethers might represent the main electron donors for desulfitobacteria in terrestrial ecosystems due to their abundancy in comparison with other electron donors that can be used by them, such as organic acids (pyruvate, lactate and formate). Furthermore, their presence in each of the sampled soils verifies the previously suggested ubiquity of this genus in terrestrial ecosystems (Lanthier et al., 2001).

4.4 The role of *Desulfitobacterium* spp. in anoxic *O*-demethylating enrichment cultures

The enrichment of *Desulfitobacterium* spp. by exploitation of their methylotrophic metabolism was achieved for the first time in the present study, hinting that the *O*-demethylation of phenyl methyl ethers might play an important role for the survival of these bacteria in natural habitats such as forest and grassland topsoils. This hypothesis is reflected in the enrichment of *Desulfitobacterium* spp. 16S rRNA gene copies up to 10% of the total bacterial 16S rRNA gene copy number in enrichment cultures, in which thiosulfate was provided as an electron acceptor for *O*-demethylation. This finding indicates the participation of *Desulfitobacterium* spp. in the *O*-demethylation of phenyl methyl ethers in addition to mostly acetogenic bacteria that were identified by community analyses. However, an enrichment could only be observed during the early sub-cultivation steps of each enrichment culture. A loss of 16S rRNA gene copies of desulfitobacteria was observed at later sub-cultivation stages, concomitant with the increase of the number of OTUs belonging to acetogenic bacteria such as *Alkalibaculum* spp., *Clostridium* spp., *Sporobacterium* spp., *Sporomusa* spp. and *Tissierella* spp. This trend was observed in every enrichment culture, regardless of the electron acceptor that was used for enrichment. A possible explanation for the initial enrichment of desulfitobacteria is the existence of an advantage over acetogens and other *O*-demethylating organisms during the early sub-cultivation steps. The metabolism of *Desulfitobacterium* spp., described as versatile (Villemur et al., 2006), might allow them to couple the *O*-demethylation of phenyl methyl ethers not only

to the electron acceptor provided in the medium (thiosulfate), but also to other electron acceptors that may originate from the soil inoculum and that might still be available during the early sub-cultivation steps (e.g. nitrate, Fe(III), organic acids), favoring a faster adaptation to the culturing conditions. Moreover, the presence of additional carbon and/or energy sources as well as of useful supplements (trace elements, vitamins) provided by the soil used as starting material for the enrichment cannot be excluded. These additional substrates may allow *Desulfitobacterium* spp. to choose between different survival strategies when competing for growth substrates within microbial consortia. The presence of residual oxygen that could not be purged from the soil inoculum during its anaerobization might have inhibited the growth of acetogens, further promoting the enrichment of *Desulfitobacterium* spp. Even though desulfitobacteria are described as strictly anaerobic bacteria (Villemur et al., 2006), they can tolerate small amounts of oxygen, whereas acetogens such as *Acetobacterium dehalogenans* are much more sensitive to oxygen, as could be observed in laboratory strains (unpublished data). The soil inoculum is physically present in the enrichment cultures during the first and second sub-cultivation step, and is diluted to exctinction after the second transfer step. Its presence and absence correlate with the enrichment and outcompetition of desulfitobacteria, respectively, and might therefore play a key role in this process. Hence, the "late blooming" of acetogens may be explained by the absence of residual oxygen that inhibits their growth, and the absence of additional carbon and energy sources for desulfitobacteria that may favor initial growth of the latter organisms. The fact that acetogens have specialized on methylotrophic metabolism, while desulfitobacteria can be considered metabolic allrounders, may also play a role in the outcompetition process. The presence of CO_2 during the enrichment might further stimulate the thriving of acetogens in later sub-cultivation steps.

4.5 The use of FTHFS as a marker gene for the detection of *Desulfitobacterium* spp. in environmental compartments

Species belonging to the genus *Desulfitobacterium* were reported to contain different numbers of heterogeneous 16S rRNA genes (Villemur et al., 2007). The amount can vary from eight in *D. metallireducens* to three in *D. hafniense* strains TCP-A and PCP-1. Therefore, an accurate enumeration of *Desulfitobacterium* cells in environmental samples by qPCR analyses is hampered by the presence of multiple differing 16S rRNA gene copies of the enriched *Desulfitobacterium* species. Thus, the establishment of a new marker gene for the detection of desulfitobacteria by qPCR was attempted. The formyltetrahydrofolate synthetase (FTHFS) gene, the protein product of which mediates the reversible ATP-dependent activation of formate

and its ligation to tetrahydrofolate (Drake et al., 2006; Ragsdale & Pierce, 2008), has been considered to be a suitable marker gene for the detection of methylotrophic bacteria (e.g. acetogens) in environmental compartments. Especially for acetogens, which comprise a heterogeneous group of bacteria, the use of a functional gene as a marker is advantageous for their detection, as these organisms are not necessarily phylogenetically related to each other. Expanding the use of the FTHFS gene as a marker gene for desulfitobacteria would prove useful for the quantification of these organisms in relation to the methylotrophic community. However, most *Desulfitobacterium* genomes contain two FTHFS gene copies that share approximately 65% identity at the amino acid level. In order to assess which out of these two gene copies might be involved in phenyl methyl ether metabolism, the expression of these genes was evaluated under different growth conditions with the aim of choosing the appropriate gene copy as a marker gene for the qPCR detection of desulfitobacteria in environmental samples. In *D. hafniense* DCB-2, both FTHFS gene copies (Dhaf_0149 and Dhaf_0555) were expressed under four different growth conditions. However, the signal intensity of Dhaf_0149 exceeded that of Dhaf_0555, indicating a higher transcript level of the first copy in growing cells.

The presence of multiple gene copies for gene products involved in the Wood-Ljungdahl pathway, including FTHFS, but also the CO dehydrogenase:acetyl-CoA synthase complex, has been described before for some acetogens (Gagen et al., 2010). From studies in the methanogen *Carboxydothermus hydrogenoformans* it was hypothesized that multiple CO dehydrogenase copies might play a role in different metabolic pathways (Wu et al., 2005). Since FTHFS participates in various biosynthesis pathways such as those of amino acids and purines (Whitehead et al., 1988), it could be argued that Dhaf_0149 might be responsible for nucleotide and amino acid metabolism, and that Dhaf_0555 is part of the methyl branch of the Wood-Ljungdahl pathway, according to the putative function of the neighbor genes (**Figure 3.12**). This hypothesis does not exclude the presence of both gene copies, Dhaf_0149 and Dhaf_0555, in the absence of phenyl methyl ethers, such as during growth on pyruvate, as it was shown in **Figure 3.13**. In *Desulfitobacterium* spp., pyruvate is converted to acetyl-CoA and CO_2 via a pyruvate:flavodoxin/ferredoxin oxidoreductase (Dhaf_0054 and Dhaf_4766, respectively) or to formate via a pyruvate:formate lyase (Dhaf_0366, Dhaf_1246 and Dhaf_4905). The CO_2 resulting from pyruvate conversion could be reduced via the methyl branch of the Wood-Ljungdahl pathway to a methyl group in CH_3-FH_4, which might serve as intermediate in the synthesis of amino acids, purines and nucleotides. The fact that a Dhaf_0555 homolog is not present in *D. dichloroeliminans*, which harbors putative demethylase operons in its genome (see APPENDIX section, **Figure 6.9**), contradicts the hypothesis of both gene products being

active in different metabolic pathways. Parting from the hypothesis that *D. dichloroeliminans* is capable of *O*-demethylation, which could not be proved in this study due to poor growth of this organism, the Dhaf_0149 copy and its homologs must encode multifunctional enzymes that are active in multiple metabolic pathways at the same time. This would explain the higher gene expression of Dhaf_0149 under all growth conditions, which was still stronger than the one of Dhaf_0555 even when only 1/10 of total RNA was used for the detection of the former copy.

In contrast to the Dhaf_0555 gene copy, homologs of the Dhaf_0149 gene copy in *D. hafniense* DCB-2 could be identified in all the *Desulfitobacterium* genomes sequenced so far. The Dhaf_0149 copy shares a 60-70% identity at the amino acid level with the FTHFS enzyme of acetogenic bacteria, as revealed by BLAST P alignments (data not shown), and features distinct amino acid residues that can specifically be targeted by primers on the DNA level (see APPENDIX section, **Figure 6.2**). For this reason, and in order to possibly detect all desulfitobacteria present in environmental samples, the Dhaf_0149 gene copy in *D. hafniense* DCB-2 and its homologs in the remaining desulfitobacteria were chosen to serve as marker genes for the detection of these organisms in a newly designed qPCR assay. As only the Dhaf_0149 gene copy in *D. hafniense* DCB-2 and its homologs in other *Desulfitobacterium* spp. (see **Table 3.9**) are detected, the assay provides a new method for quantification of desulfitobacteria in environmental samples and habitats. Since in this assay only one gene copy is amplified per *Desulfitobacterium* genome, the quantified gene copy number equals the *Desulfitobacterium* cell number, as opposed to the 16S rRNA qPCR detection, in which different amounts of 16S rRNA gene copies detected per *Desulfitobacterium* genome can prove fastidious for an accurate enumeration. The cell number derived from normalizing the *Dsf* 16S rRNA gene copy number to 5 copies per genome is on average 3 times higher than the cell number derived from *Dsf* FTHFS copy numbers. This deviation probably reflects the different number of *Dsf* 16S rRNA gene copies in each *Desulfitobacterium* genome. However, since up to the date no primer pair can accurately cover the amplification of the entirety of bacterial FTHFS gene copies due to their diversity, it is hard to evaluate the relative abundance of *Desulfitobacterium* spp. among other demethylating bacteria such as acetogens by this assay. The FTHFS assay may therefore be used for a direct quantification of *Desulfitobacterium* cell numbers, while the 16S rRNA assay may still be used to determine the relative abundance of these bacteria in a mixed community.

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6 APPENDIX

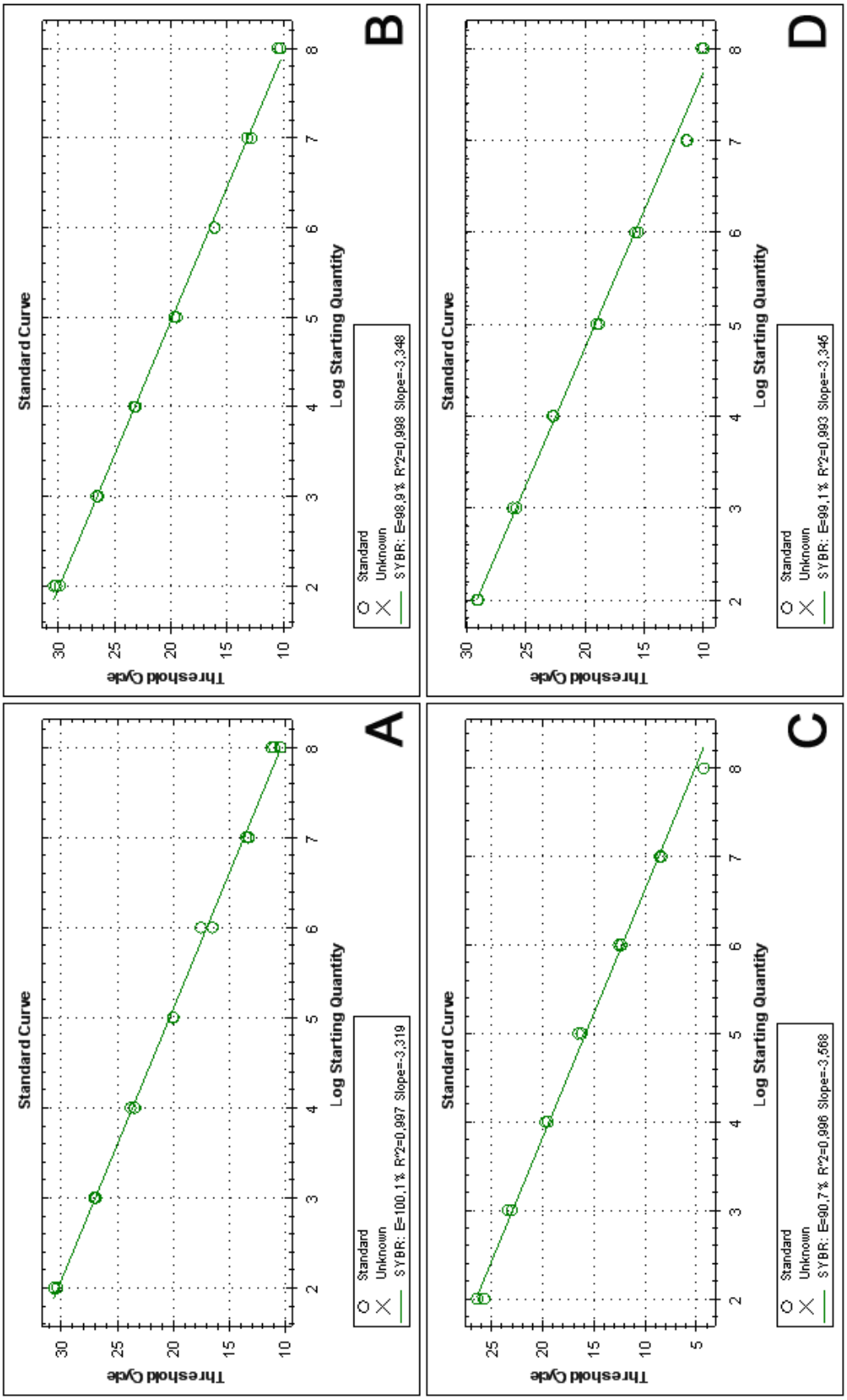

Figure 6.1: Typical standard curves and reaction efficiencies of universal (A) and *Desulfitobacterium* spp. (B) 16S rRNA, total (C) and *Desulfitobacterium* spp. FTHFS (D) qPCR assays.

Figure 6.2: Amino acid sequence alignment of Dhaf_0149 and its homologs in other *Desulfitobacteirum* spp. versus the FTHFS enzyme of acetogenic and non-acetogenic bacteria. The amino acid residues labeled yellow were used for the design of specific primers.

```
M. thermoacetica      ----MSKVPSDIEI--AQAAKMKPVMELARGLGIQEDEVELYGKYKAKISLDVYRRLKDK     54
A. woodii             -----MGFKSDIEI--AQEATPQDIREIAKKLGLTEDDLDLYGKYKAKVDYNLLKKST-G     52
E. cellulosolvens     -----MGYKSDIEI--AQECVMKPITEIAQEAGISEEYLECYGKYKAKISDKLMDHMD-R     52
S. ovata              -------MKSDVEI--AQEAKMNPIAEVAKELMIPAEELELYGNYKTKVSLATWERIKGK     51
C. aceticum           -----MSFKSDIEI--AQEATPQDIREVASKLNLTENDIELYGKYKAKVDYNLLKQDNGG     53
B. azotoformans       MSVQSKKVKSDIEI--AQEAVMKPIKEIAEGLNLREDEWEPFGHYKAKISLDVMKRLQNA     58
D. orientis           MAWDATKL-KDWQIAEEAEKSMPTVEELIEKLSLRKEEIIPYGKTPKVDFLKMMERLGDK     59
D. hafniense DCB-2    -------MKTDIEI--AQEATMKPITEIAQGLDLLEDEIELYGKYKAKVNFSAWERLKDK     51
D. hafniense DP7      -------MKTDIEI--AQEATMKPITEIAQGLDLLEDEIELYGKYKAKVNFSAWERLKDK     51
D. hafniense TCE1     -------MKTDIEI--AQEATMKPITEIAQGLDLLEDEIELYGKYKAKVNFSAWERLKDK     51
D. hafniense TCP-A    -------MKTDIEI--AQEATMKPITEIAQGLDLLEDEIELYGKYKAKVNFSAWERLKDK     51
D. hafniense PCP-1    -------LKTDIEI--AQEATMKPITEIAQGLDLLEDEIELYGKYKAKVNFSAWERLKDK     51
D. hafniense Y51      -------MKTDIEI--AQEATMKPITEIAQGLDLLEDEIELYGKYKAKVNFSAWERLKDK     51
D. dehalogenans       -------MKTDIEI--AQEATMKPITEVAQGLDLLEDEIELYGKYKAKVNFSAWERLKDQ     51
D. dichloroeliminans  -------MKTDIEI--AQESTMKPIMDVAQGLELLDDEIELYGKYKAKINLSAWDRLKDR     51

M. thermoacetica      PDGKLILVTAITPTPAGEGKTTTSVGLTDALARLGKRVMVCLREPSLGPSFGIKGGAAGG     114
A. woodii             KKARLILTTAINPTPAGEGKTTTTIGVADGLSRIGKNTLVALREPSLGPVFGVKGGAAGG     112
E. cellulosolvens     PNGKLVLVTAINPTPAGEGKTTTSIGLIDALSRLGKHPVGALREPSMGPVFGVKGGAAGG     112
S. ovata              PNGKLILVTAINPTPAGEGKTTTTVGLGDSLRRKGKKVVIALREPSLGPCFGVKGGAAGG     111
C. aceticum           KKAKLILTTAINPTPAGEGKTTTTIGTSDALSRLGKKTIVALREPSLGPVFGVKGGAAGG     113
B. azotoformans       PDGKVILVTSINPTPAGEGKSTVTVGLGQALNKIGHKTIVALREPSLGPTMGIKGGASGG     118
D. orientis           PDGKYIEVTAITPTPLGEGKTTTTLGLIEGLAKRGKNVGGAVRQPSGGPTMNIKGTAAGG     119
D. hafniense DCB-2    PDAKLILVTAINPTPAGEGKTTTTVGLGQAMSKIGKNAMIALREPSLGPCFGVKGGAAGG     111
D. hafniense DP7      PDAKLILVTAINPTPAGEGKTTTTVGLGQAMSKIGKNAMIALREPSLGPCFGVKGGAAGG     111
D. hafniense TCE1     PDAKLILVTAINPTPAGEGKTTTTVGLGQAMSKIGKNAMIALREPSLGPCFGVKGGAAGG     111
D. hafniense TCP-A    PDAKLILVTAINPTPAGEGKTTTTVGLGQAMSKIGKNAMIALREPSLGPCFGVKGGAAGG     111
D. hafniense PCP-1    PDAKLILVTAINPTPAGEGKTTTTVGLGQAMSKIGKNAMIALREPSLGPCFGVKGGAAGG     111
D. hafniense Y51      PDAKLILVTAINPTPAGEGKTTTTVGLGQAMSKIGKNAMIALREPSLGPCFGVKGGAAGG     111
D. dehalogenans       PDGKLILVTAINPTPAGEGKTTTTVGLGQAMSKIGKKAMIALREPSLGPCFGVKGGAAGG     111
D. dichloroeliminans  HDGQLILVTAINPTPAGEGKTTTTVGLGQAMAKLGKKAMIALREPSLGPCFGIKGGAAGG     111

M. thermoacetica      GYAQVVPMEDINLHFTGDIHAVTYAHNLLAAMVDNHLQQG-------------NVLNIDP     161
A. woodii             GYAQVVPMEDINLHFTGDFHAIGAANNLLAAMLDNHIKQG-------------NELKIDA     159
E. cellulosolvens     GYAQVVPMEDINLHFTGDMHAIGAANNLLAAMVDNSIQQG-------------NPLNIDP     159
S. ovata              GYAQVVPMEDINLHFTGDFHAITTAHNLLAAVIDNHLHHG-------------NALGIDS     158
C. aceticum           GYAQVIPMEDINLHFTGDFHAIGAANNLLAAMLDNHINHG-------------NQLGIDN     160
B. azotoformans       GYSQVMPMEDINLHFTGDFHAITTANNALAAFLDNHIHQG-------------NECQIDT     165
D. orientis           GNALLIPMTEFSLGLTGDINDIMNAHNLCMVALNARMQHEANYTDEELAKRGLKRLDIDP     179
D. hafniense DCB-2    GYAQVVPMEDINLHFTGDFHAITSTHNLLAALLDNHIQQG-------------NLLNIDP     158
D. hafniense DP7      GYAQVVPMEDINLHFTGDFHAITSTHNLLAALLDNHIQQG-------------NLLNIDP     158
D. hafniense TCE1     GYAQVVPMEDINLHFTGDFHAITSTHNLLAALLDNHIQQG-------------NLLNIDP     158
D. hafniense TCP-A    GYAQVVPMEDINLHFTGDFHAITSTHNLLAALLDNHIQQG-------------NLLNIDP     158
D. hafniense PCP-1    GYAQVVPMEDINLHFTGDFHAITSTHNLLAALLDNHIQQG-------------NLLNIDP     158
D. hafniense Y51      GYAQVVPMEDINLHFTGDFHAITSTHNLLAALLDNHIQQG-------------NLLNIDP     158
D. dehalogenans       GYAQVVPMEDINLHFTGDFHAITSTHNLLAALLDNHIQQG-------------NLLNIDP     158
D. dichloroeliminans  GYAQVVPMEDINLHFTGDFHAITSTHNLLAALLDNHIQQG-------------NLLNIDS     158
```

Figure 6.3: Amino acid sequence alignment of Dhaf_0555 and its homologs in other *Desulfitobacteirum* spp. versus the FTHFS enzyme of acetogenic and non-acetogenic bacteria. The amino acid residues labeled yellow were used for the design of specific primers.

```
M. thermoacetica      KIAKIATEIYGADGVNYTAEADKAIQRYES-LGYGNLPVVMAKTQYSFSDDMTKLGRPRN     508
A. woodii             KIETIATRIYGADGVDFTPAAAKEMDRLTA-LGFDKVPICMAKTQYSLTDDATKLGRPTG     507
E. cellulosolvens     KLNAIVQKVYGGARAELTSNAKNQAKKLTA-MGYENVPICMAKTQYSLTDDPKKLGAPKD     511
S. ovata              KIAAIATKIYGADGVNYTAAAEKTSKELTA-LGFDKTPICMAKTQYSLSDDMSKMGRPTG     504
C. aceticum           KITAIAQKIYGADNADFTPAALKEIDRLTK-LGFDKLPICMAKTQYSLTDNQNVLGRPTG     507
B. azotoformans       KIETIAKKVYGAVAVDFIPKVRKQIEEYKS-LGWDKLPVCMAKTQYSLSDDPAKLGRPEG     512
D. orientis           RVELIAKEVYGADGVDWSPEAEAKAKRFESDPHYADFSTMMVKTHLSLSHDPTLKGVPKG     534
D. hafniense DCB-2    KITAIATKIYGADGVDFIGSSTKDIEGIES-IGYRNIPVCMAKTQYSLSDDQKKLGRPTG     507
D. hafniense DP7      KITAIATKIYGADGVDFIGSSTKDIEGIES-IGYRNIPVCMAKTQYSLSDDQKKLGRPTG     507
D. hafniense TCE1     KITAIATKIYGADGVDFIGSSTKDIEGIES-IGYRNIPVCMAKTQYSLSDDQKKLGRPTG     507
D. hafniense TCP-A    KITAIATKIYGADGVDFIGSSTKDIEGIES-IGYRNIPVCMAKTQYSLSDDQKKLGRPTG     507
D. hafniense PCP-1    KITAIATKIYGADGVDFIGSSTKDIEGIES-IGYRNIPVCMAKTQYSLSDDQKKLGRPTG     507
D. hafniense Y51      KITAIATKIYGADGVDFIGSSTKDIEGIES-IGYRNIPVCMAKTQYSLSDDQKKLGRPTG     507
D. dehalogenans       KITAIATKIYGADGVDFIGSSAKDIENIES-IGYRNIPVCMAKTQYSLSDDQKKLGRPTG     507

M. thermoacetica      FTITVREVRLSAGAGFIVPITGAIMTMPGLPKRPAACNIDIDA-DGVITGLF    559
A. woodii             FKITVRQLTISAGAGFIIALTGEIMKMPGLPKVPAAEKIDVDE-NGVIAGLF    558
E. cellulosolvens     FVVTVRNLKISAGAGFIVALTGDIMTMPGLPKHPAAMDIDVDE-NGKITGLF    562
S. ovata              FTITVREIRVAAGAGFLVAITGDIMTMPGLPKEPSAIKMDIDN-SGKIVGLF    555
C. aceticum           FNITVRQVKVSAGAGFLVALTGEIMTMPGLPKVPSAEKIDVDE-SGVITGLF    558
B. azotoformans       FTITVRELRPSIGAGFLVALTGDVMTMPGLPKKPAALNMDVDA-DGKAIGLF    563
D. orientis           WRLPIRDVLVYGGAKFLCPMAGAISLMPGTSSDPAYRRIDVDTKTGKVSGLF    586
D. hafniense DCB-2    FRISIRSVKVSAGAGFAVALTGDIMTMPGLPKVPAAESIDVDN-TGRISGLF    558
D. hafniense DP7      FRISIRSVKVSAGAGFAVALTGDIMTMPGLPKVPAAESIDVDN-TGRISGLF    558
D. hafniense TCE1     FRISIRSVKVSAGAGFAVALTGDIMTMPGLPKVPAAESIDVDN-TGRISGLF    558
D. hafniense TCP-A    FRISIRSVKVSAGAGFAVALTGDIMTMPGLPKVPAAESIDVDN-TGRISGLF    558
D. hafniense PCP-1    FRISIRSVKVSAGAGFAVALTGDIMTMPGLPKVPAAESIDVDN-TGRISGLF    558
D. hafniense Y51      FRISIRSVKVSAGAGFAVALTGDIMTMPGLPKVPAAESIDVDN-TGRISGLF    558
D. dehalogenans       FRISIRSVKVSAGAGFAVALTGDIMTMPGLPKVPAAESIDVDN-TGKISGLF    558
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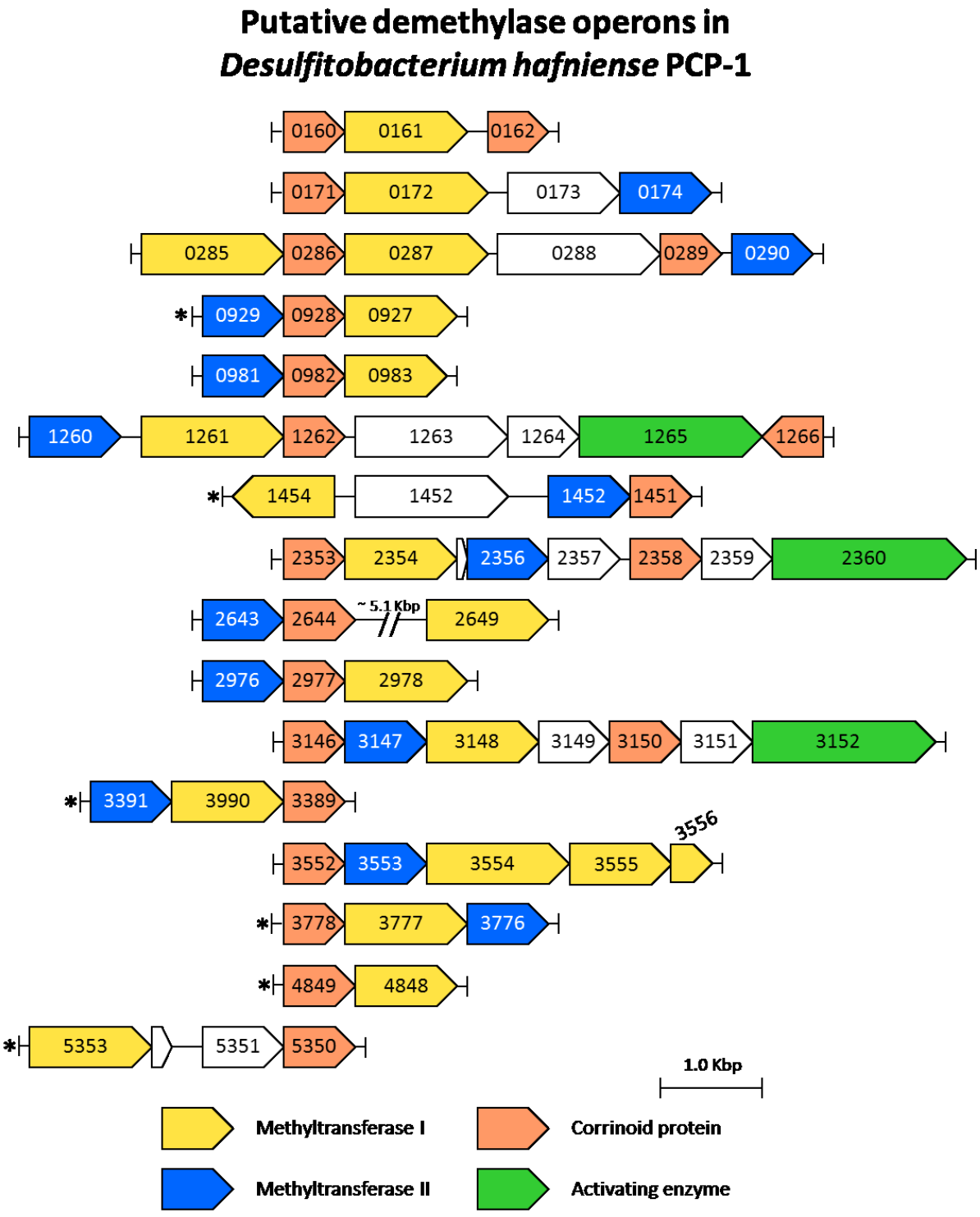

Figure 6.4: Physical gene map of putative demethylase systems in *D. hafniense* PCP-1. The corresponding genes were identified via the IMG server. Each locus tag number is preceded by the prefix "A37YDRAFT_". Asterisks indicate an inverse orientation of all genes included in the cluster compared to the IMG output.

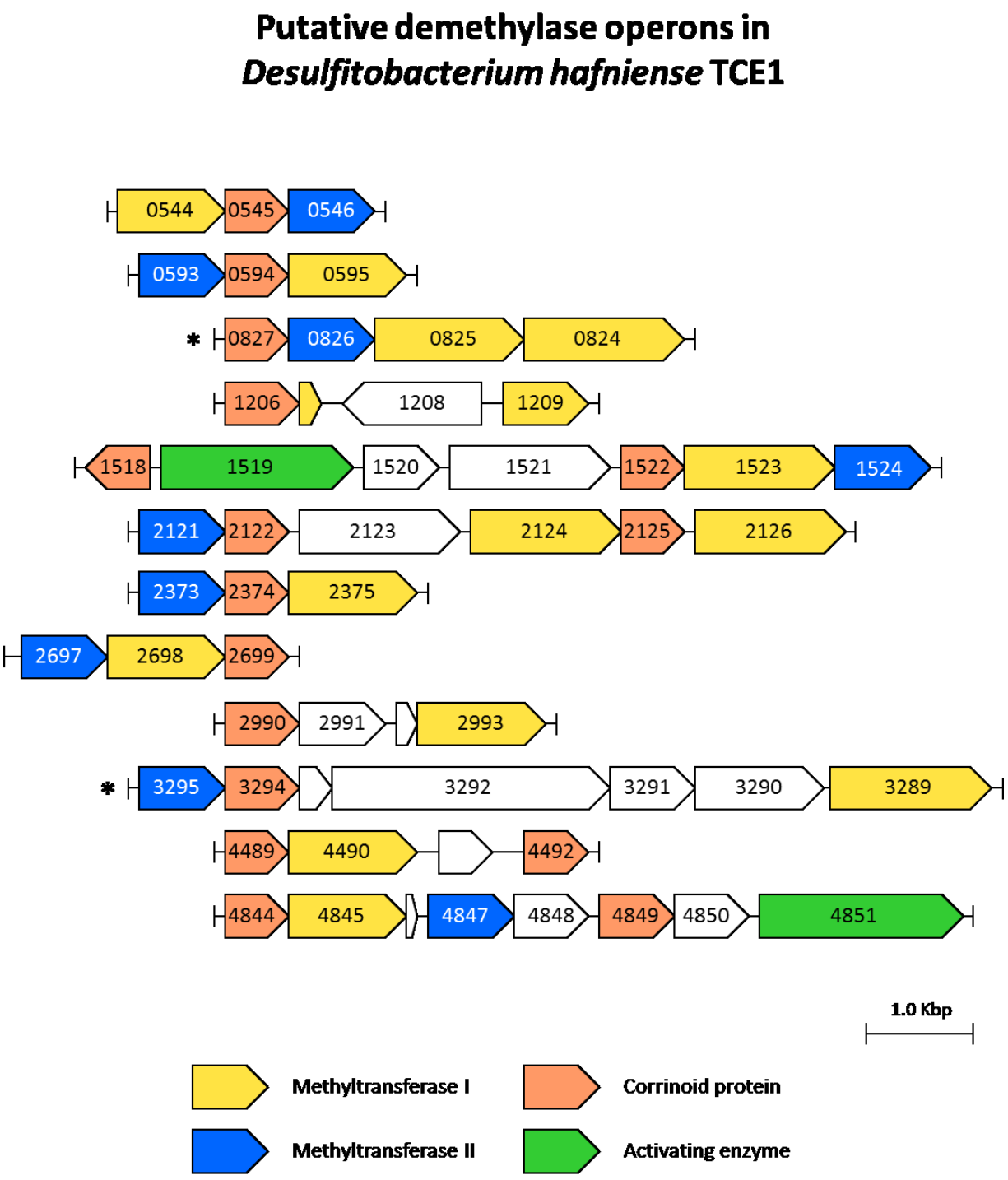

Figure 6.5: Physical gene map of putative demethylase systems in *D. hafniense* TCE1. The corresponding genes were identified via the IMG server. Each locus tag number is preceded by the prefix "DeshaDRAFT_". Asterisks indicate an inverse orientation of all genes included in the cluster compared to the IMG output.

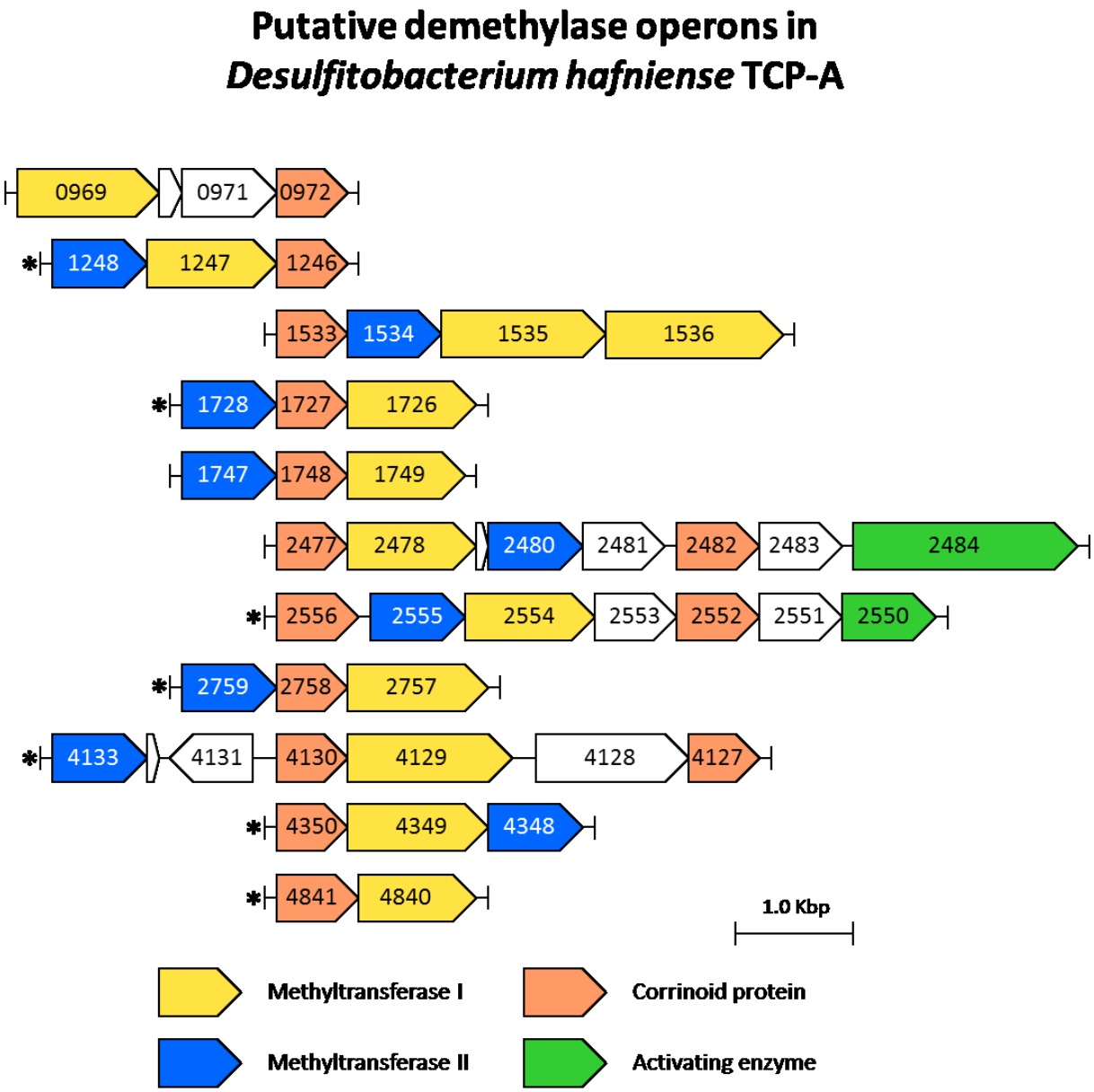

Figure 6.6: Physical gene map of putative demethylase systems in *D. hafniense* TCP-A. The corresponding genes were identified via the IMG server. Each locus tag number is preceded by the prefix "DeshafDRAFT_". Asterisks indicate an inverse orientation of all genes included in the cluster compared to the IMG output.

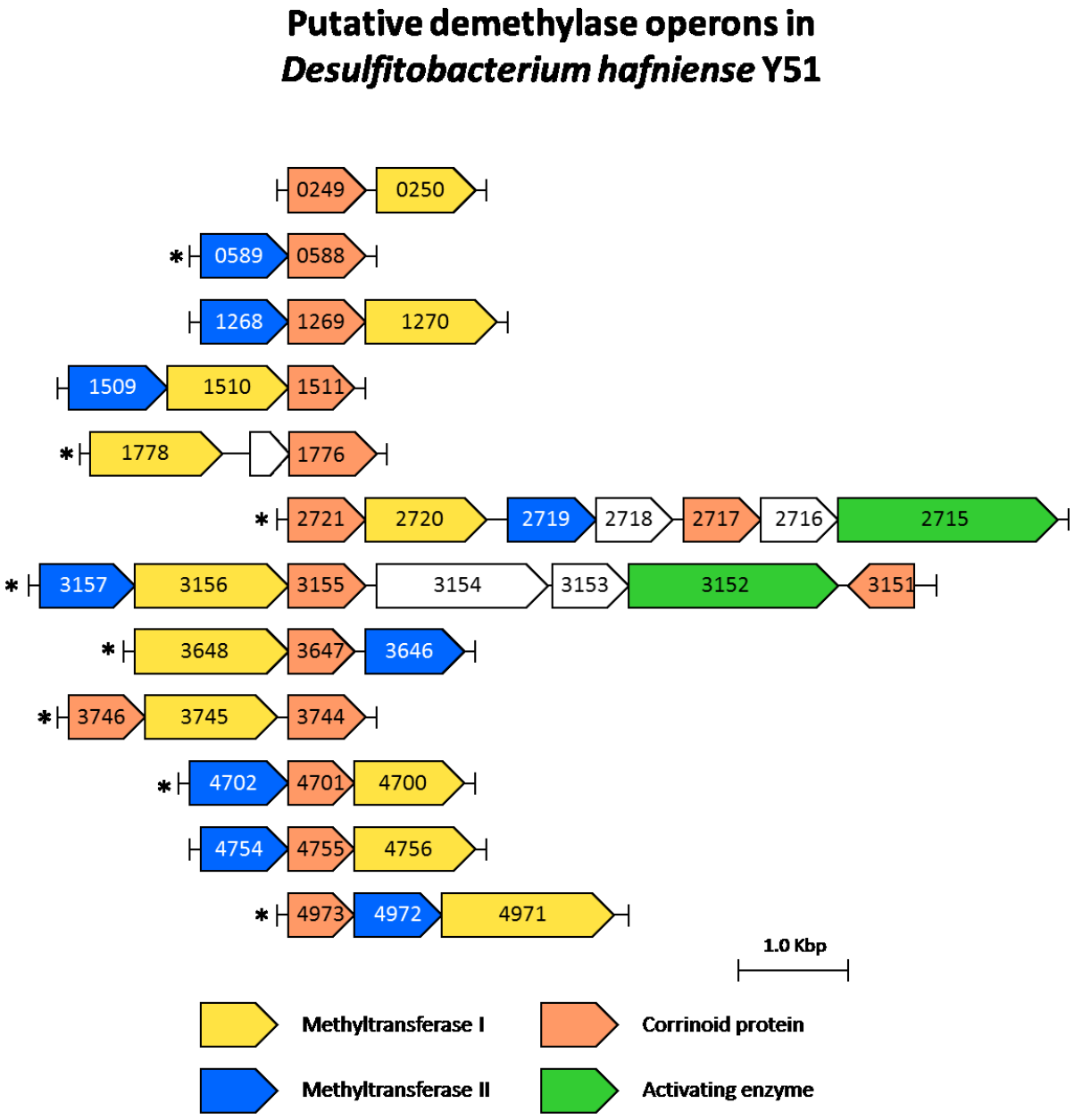

Figure 6.7: Physical gene map of putative demethylase systems in *D. hafniense* Y51. The corresponding genes were identified via the IMG server. Each locus tag number is preceded by the prefix "DSY". Asterisks indicate an inverse orientation of all genes included in the cluster compared to the IMG output.

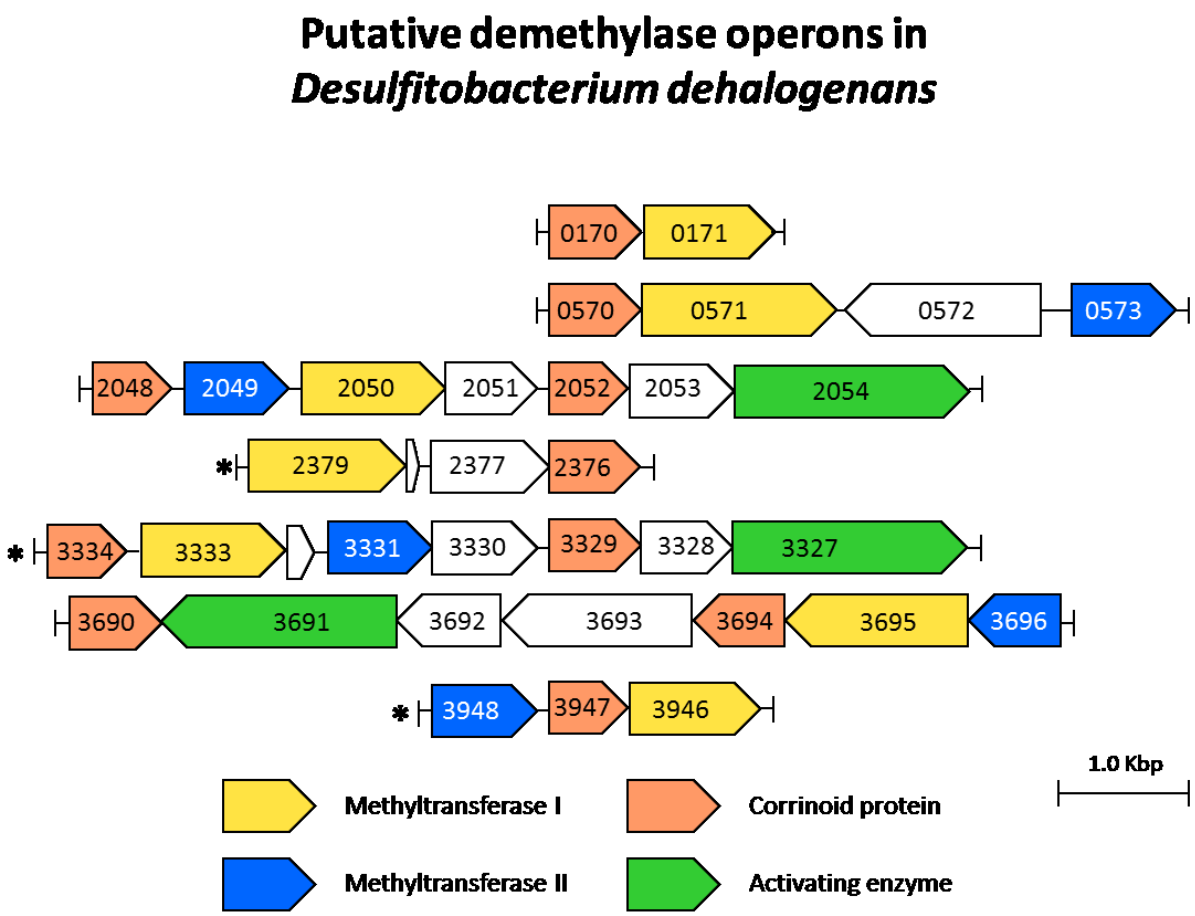

Figure 6.8: Physical gene map of putative demethylase systems in *D. dehalogenans*. The corresponding genes were identified via the IMG server. Each locus tag number is preceded by the prefix "Desde_". Asterisks indicate an inverse orientation of all genes included in the cluster compared to the IMG output.

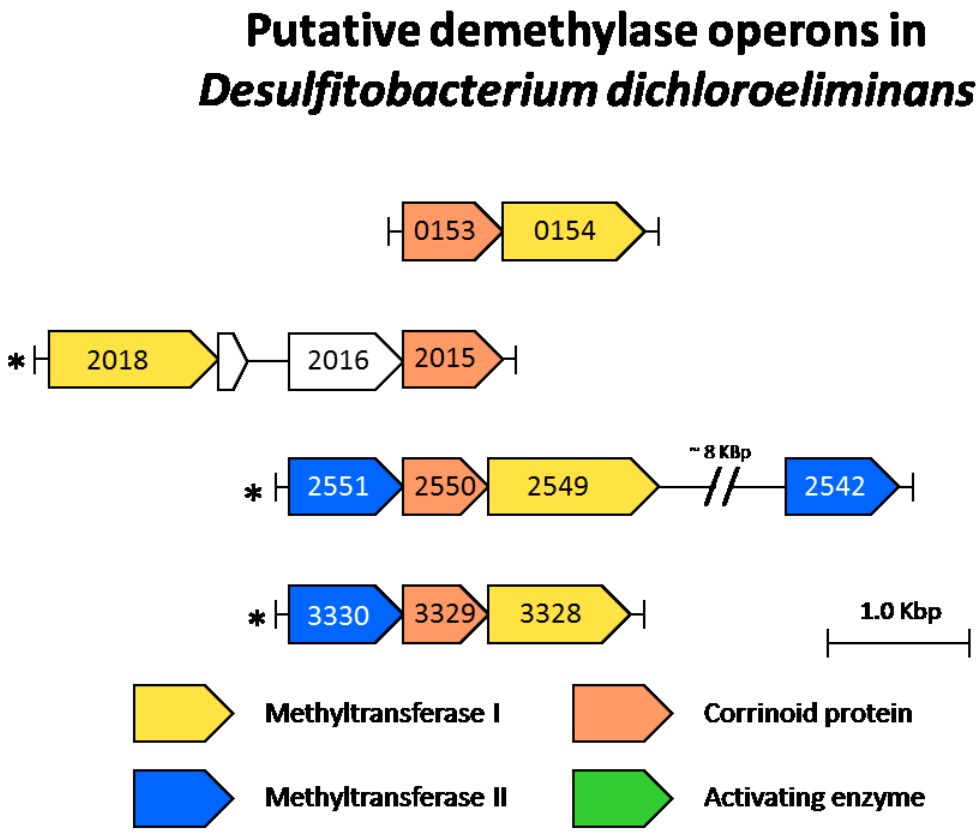

Figure 6.9: Physical gene map of putative demethylase systems in *D. dichloroeliminans*. The corresponding genes were identified via the IMG server. Each locus tag number is preceded by the prefix "Desdi_". Asterisks indicate an inverse orientation of all genes included in the cluster compared to the IMG output.

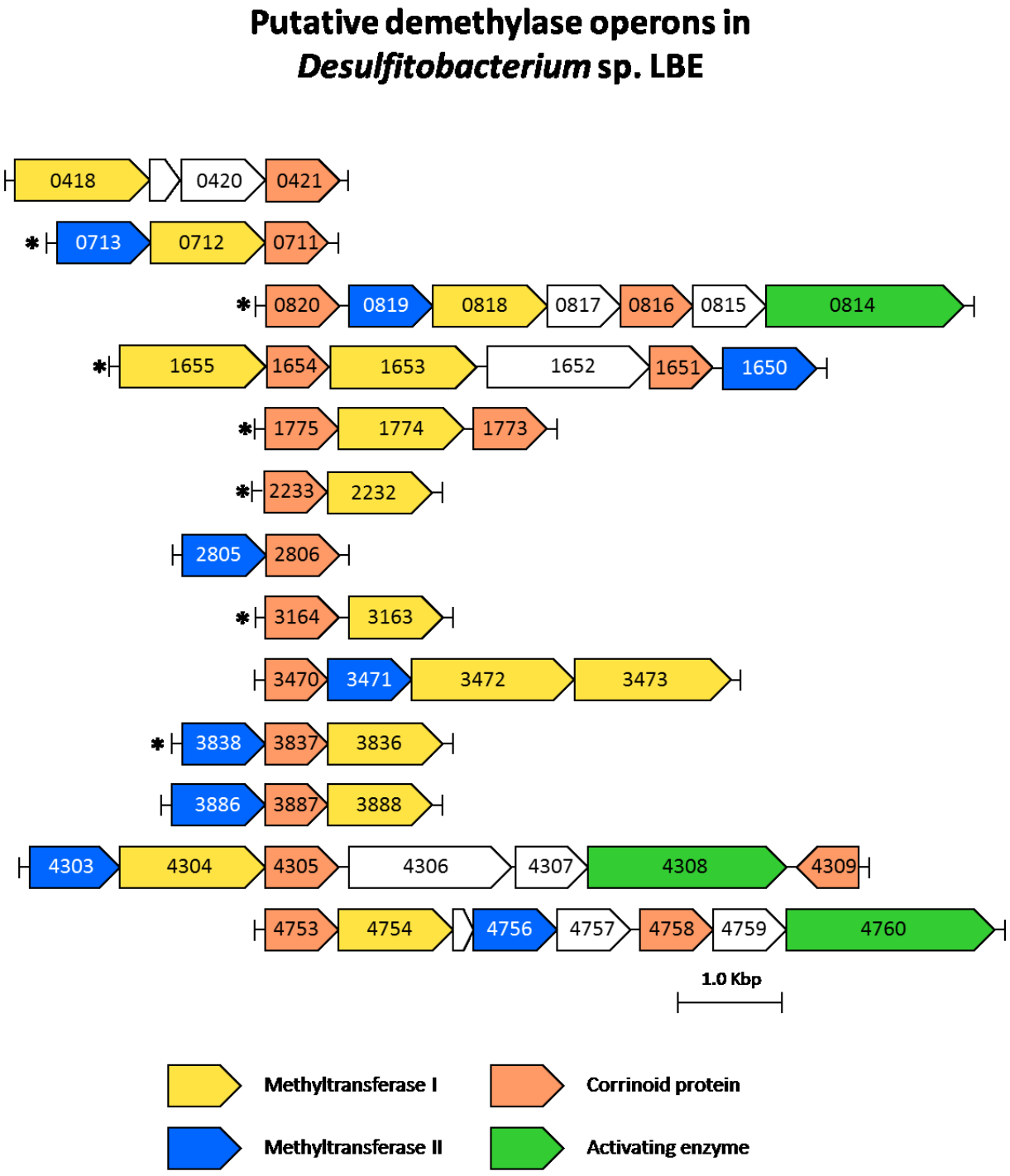

Figure 6.10: Physical gene map of putative demethylase systems in *Desulfitobacterium* sp. LBE. The corresponding genes were identified via the IMG server. Each locus tag number is preceded by the prefix "DesLBEDRAFT_". Asterisks indicate an inverse orientation of all genes included in the cluster compared to the IMG output.

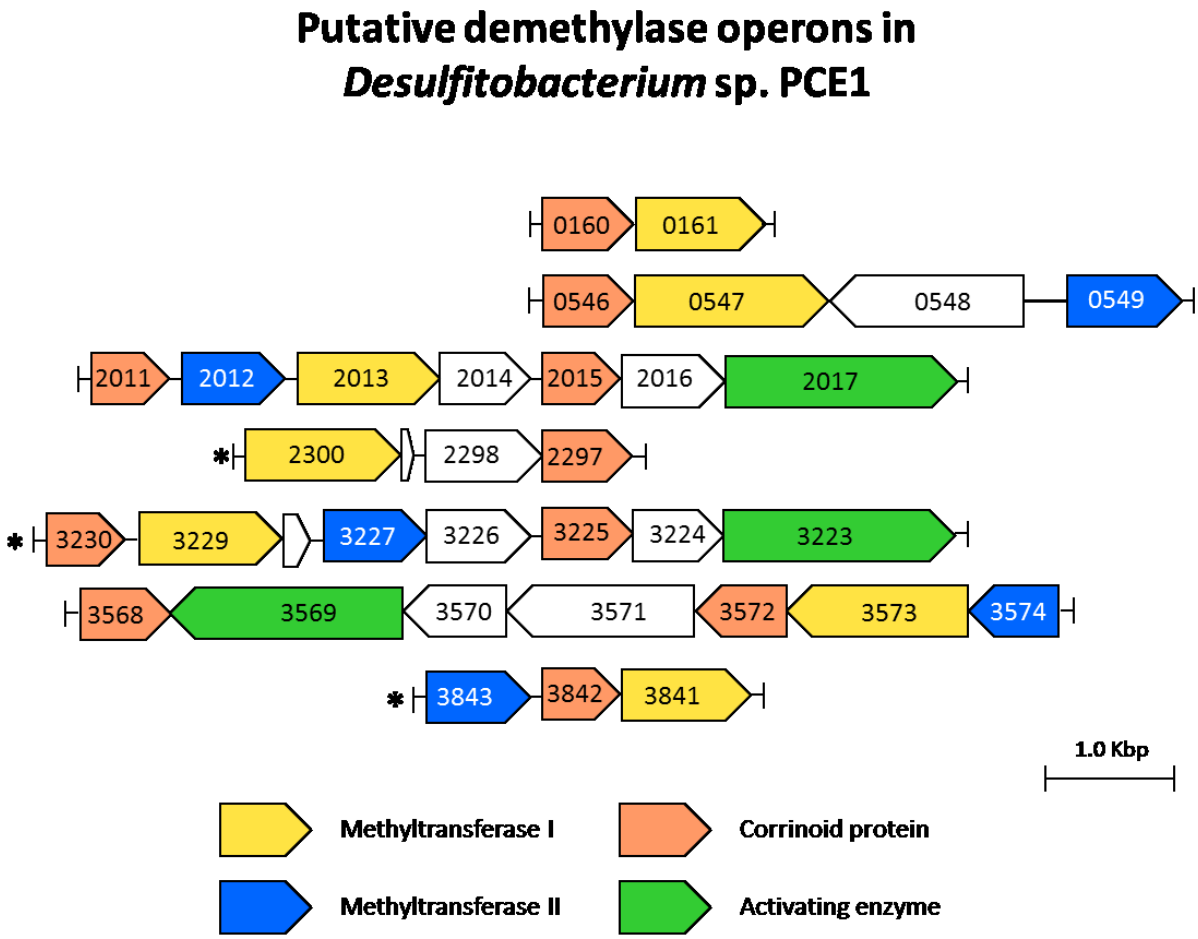

Figure 6.11: Physical gene map of putative demethylase systems in *Desulfitobacterium* sp. PCE1. The corresponding genes were identified via the IMG server. Each locus tag number is preceded by the prefix "DesPCE1DRAFT_". Asterisks indicate an inverse orientation of all genes included in the cluster compared to the IMG output.

Table 6.1: Relative abundance of *Desulfitobacterium* spp. 16S rRNA and FTHFS gene copies in the first (#1) and second (#2) biological replicate of each enrichment culture amended with syringate and thiosulfate. The maximum relative amounts for each enrichment are highlighted in bold font. Abbreviation: n.d., not determined.

	Cambisol				**Luvisol**				**Gleysol**				**Pelosol**				**Podsol**			
Sub-cultivation	**16S rRNA**		**FTHFS**		**16S rRNA**		**FTHFS**		**16S rRNA**		**FTHFS**		**16S rRNA**		**FTHFS**		**16S rRNA**		**FTHFS**	
	#1	**#2**	**#1**	**#2**	**#1**	**#2**	**#1**	**#2**	**#1**	**#2**	**#1**	**#2**	**#1**	**#2**	**#1**	**#2**	**#1**	**#2**	**#1**	**#2**
1	**5.66**	3.17	**28.58**	18.61	1.32	0.90	**4.65**	0.10	3.08	0.05	12.64	0.01	3.26	0.82	5.84	0.25	0.04	1.62	0.03	0.00
2	3.44	**8.17**	3.58	**99.12**	**3.20**	2.42	2.59	**1.41**	**10.04**	1.74	**52.82**	0.59	**6.45**	**8.93**	**11.27**	**0.26**	**0.20**	**4.91**	**3.12**	**0.05**
3	0.81	2.72	0.55	92.62	0.53	0.61	0.80	0.95	2.23	**3.59**	25.52	**4.88**	0.93	4.70	0.72	0.13	0.04	1.04	0.08	0.01
4	0.21	0.42	0.02	20.74	1.36	1.48	3.01	0.01	0.11	2.76	14.96	0.66	0.90	0.18	0.18	0.14	n.d.	n.d.	n.d.	n.d.
5	0.06	0.03	0.03	14.43	0.40	**3.54**	0.96	0.00	2.72	1.50	38.12	0.22	1.09	0.10	0.43	0.16	n.d.	n.d.	n.d.	n.d.

Script 6.1: mothur batch script.

```
mothur > make.contigs(file=enrichment.files, processors=8)

mothur > summary.seqs(fasta=enrichment.trim.contigs.fasta)

mothur > screen.seqs(fasta=enrichment.trim.contigs.fasta,
group=enrichment.contigs.groups, maxambig=0, maxhomop=8, maxlength=600)

mothur > unique.seqs(fasta=enrichment.trim.contigs.good.fasta)

mothur > count.seqs(name=enrichment.trim.contigs.good.names,
group=enrichment.contigs.good.groups)

mothur > summary.seqs(count=enrichment.trim.contigs.good.count_table)

mothur > align.seqs(fasta=enrichment.trim.contigs.good.unique.fasta,
reference=silva.bacteria.fasta, flip=T)

mothur > summary.seqs(fasta=enrichment.trim.contigs.good.unique.align,
count=enrichment.trim.contigs.good.count_table)

mothur > screen.seqs(fasta=enrichment.trim.contigs.good.unique.align,
count=enrichment.trim.contigs.good.count_table,
summary=enrichment.trim.contigs.good.unique.summary, optimize=start,
end=10289, maxlength=560, minlength=430)

mothur > summary.seqs(fasta=enrichment.trim.contigs.good.unique.good.align,
count=enrichment.trim.contigs.good.good.count_table)

mothur > filter.seqs(fasta=enrichment.trim.contigs.good.unique.good.align,
vertical=T, trump=.)

mothur >
unique.seqs(fasta=enrichment.trim.contigs.good.unique.good.filter.fasta,
count=enrichment.trim.contigs.good.good.count_table)

mothur >
pre.cluster(fasta=enrichment.trim.contigs.good.unique.good.filter.unique.fa
sta, count=enrichment.trim.contigs.good.unique.good.filter.count_table,
diffs=5)

mothur >
chimera.uchime(fasta=enrichment.trim.contigs.good.unique.good.filter.unique
.precluster.fasta,
count=enrichment.trim.contigs.good.unique.good.filter.unique.precluster.cou
nt_table, dereplicate=t)

mothur >
remove.seqs(fasta=enrichment.trim.contigs.good.unique.good.filter.unique.pr
ecluster.fasta,
accnos=enrichment.trim.contigs.good.unique.good.filter.unique.precluster.uc
hime.accnos)

mothur >
classify.seqs(fasta=enrichment.trim.contigs.good.unique.good.filter.unique.
precluster.pick.fasta,
count=enrichment.trim.contigs.good.unique.good.filter.unique.precluster.uch
ime.pick.count_table, reference=trainset9_032012.pds.fasta,
taxonomy=trainset9_032012.pds.tax, cutoff=80)
```

Script 6.1: mothur batch script (continued).

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mothur >
remove.lineage(fasta=enrichment.trim.contigs.good.unique.good.filter.unique
.precluster.pick.fasta,
count=enrichment.trim.contigs.good.unique.good.filter.unique.precluster.uch
ime.pick.count_table,
taxonomy=enrichment.trim.contigs.good.unique.good.filter.unique.precluster.
pick.pds.wang.taxonomy, taxon=Chloroplast-Mitochondria-unknown-Archaea-
Eukaryota)

mothur >
phylotype(taxonomy=enrichment.trim.contigs.good.unique.good.filter.unique.p
recluster.pick.pds.wang.pick.taxonomy)

mothur > make.shared(list=enrichment.trim.c
ontigs.good.unique.good.filter.unique.precluster.pick.pds.wang.pick.tx.list
,
count=enrichment.trim.contigs.good.unique.good.filter.unique.precluster.uch
ime.pick.pick.count_table, label=1)

mothur >
classify.otu(list=enrichment.trim.contigs.good.unique.good.filter.unique.pr
ecluster.pick.pds.wang.pick.tx.list,
count=enrichment.trim.contigs.good.unique.good.filter.unique.precluster.uch
ime.pick.pick.count_table,
taxonomy=enrichment.trim.contigs.good.unique.good.filter.unique.precluster.
pick.pds.wang.pick.taxonomy, label=1)

(The three output files from classify.otu command are renamed into
enrichment.phylotypes.shared, enrichment.phylotypes.cons.tax.summary and
enrichment.phylotypes.cons.taxonomy)

mothur > sub.sample(shared=enrichment.phylotypes.shared, size=4538)

mothur > collect.single(shared=enrichment.phylotypes.1.subsample.shared,
calc=chao-invsimpson, freq=100)

mothur >
rarefaction.single(shared=enrichment.phylotypes.1.subsample.shared,
calc=sobs, freq=100)

mothur > summary.single(shared=enrichment.phylotypes.1.subsample.shared,
calc=nseqs-coverage-sobs-invsimpson, subsample=4538)

mothur > heatmap.bin(shared=enrichment.phylotypes.1.subsample.shared,
scale=log2, numotu=50)

mothur > dist.shared(shared=enrichment.phylotypes.shared, calc=thetayc-
jclass, subsample=4538)

mothur > heatmap.sim(phylip=enrichment.phylotypes.thetayc.1.lt.ave.dist)

mothur > heatmap.sim(phylip=enrichment.phylotypes.jclass.1.lt.ave.dist)
```

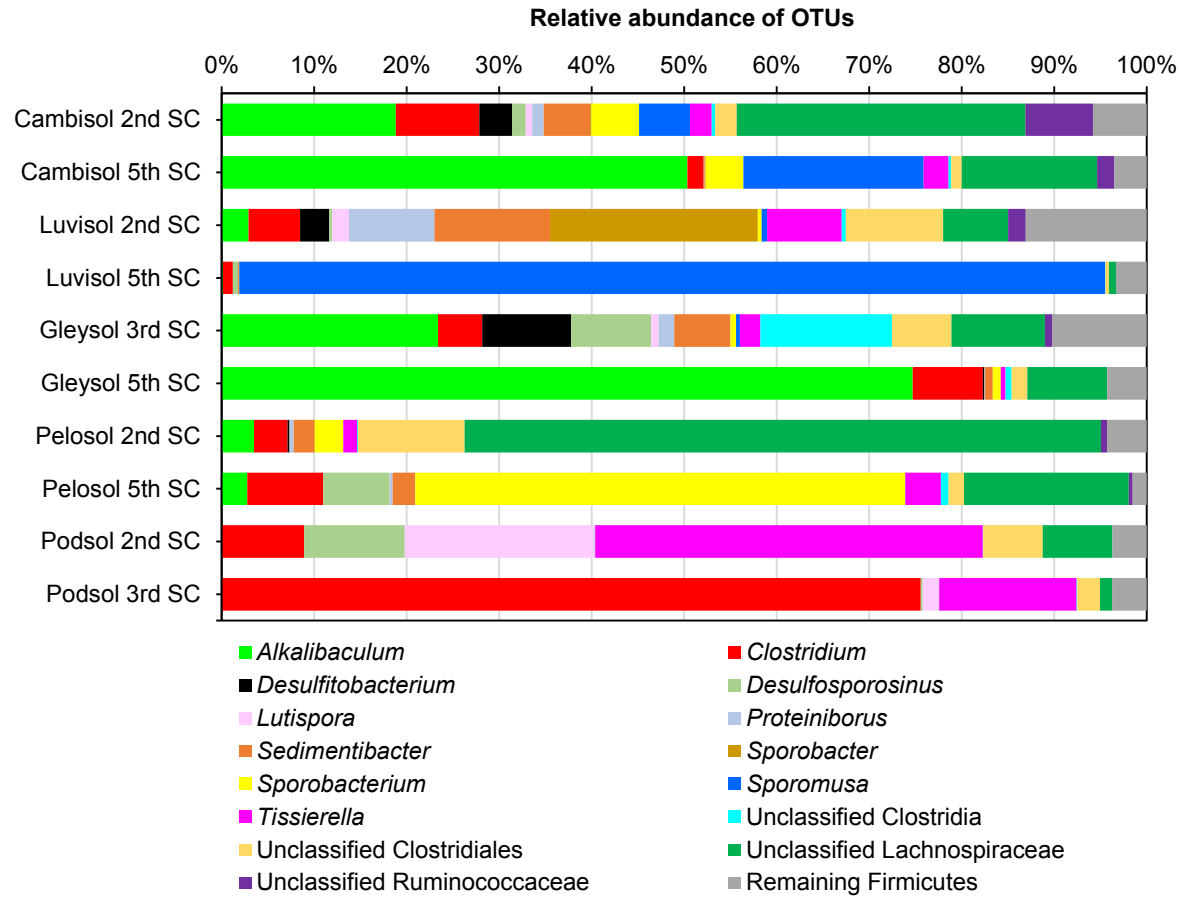

Figure 6.12: Relative abundance of OTUs belonging to Firmicutes genera in enrichment cultures amended with syringate and thiosulfate. Less representative genera that accounted for less than 1% of total Firmicutes OTUs in all samples were summarized as "Remaining Firmicutes". Abbreviation: SC, sub-culture.

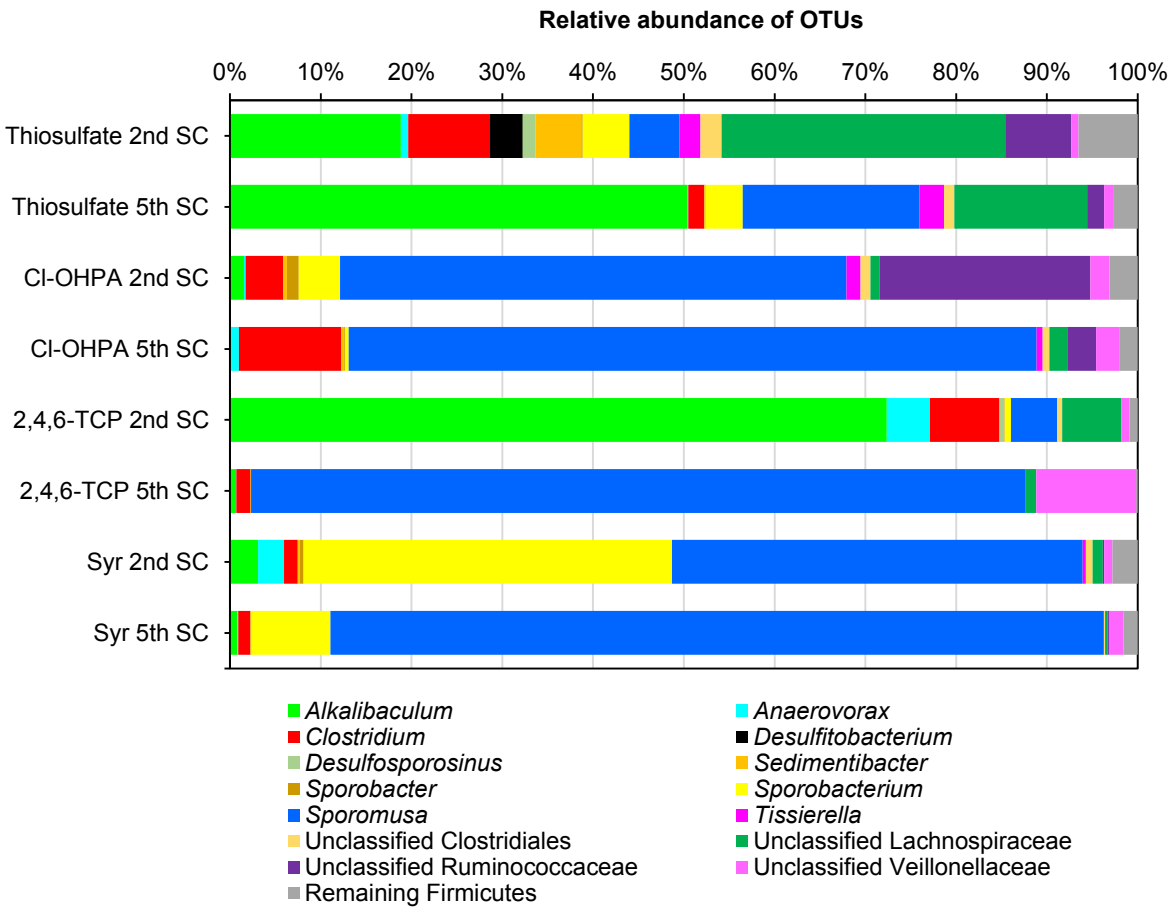

Figure 6.13: Relative abundance of OTUs belonging to Firmicutes genera in cambisol enrichment cultures amended with syringate and different electron acceptors. Less representative genera that accounted for less than 1% of total Firmicutes OTUs in all samples were summarized as "Remaining Firmicutes". Abbreviation: Cl-OHPA, 3-chloro-4-hydroxyphenylacetic acid; Syr, syringate; TCP, trichlorophenol; SC, sub-culture.

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Author's declaration of originality

I, Felix Sebastian Mingo, born on 20.11.1988 in Reus (Spain), hereby declare that I am aware of the official doctoral regulations of the Faculty of Biology and Pharmacy of the Friedrich Schiller University Jena. This thesis and the work reported herein was composed by and originated entirely from me. Information derived from the published and/or unpublished work of others has been acknowledged. The help of a doctoral advisor was not used. No commercial or monetary activities are related to the contents of this work. This dissertation has only been submitted to the Council of the Faculty of Biology and Pharmacy of the Friedrich Schiller University Jena, and not to any other university for academic examination or dissertation. This thesis is neither identical nor partially identical to any work which has been submitted as dissertation to the Friedrich Schiller University Jena or to any other university.

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Felix Sebastian Mingo

Jena, ……..

Curriculum Vitae

Name	Felix Sebastian Mingo
Date of birth	20 November 1988
Birthplace	Reus (Spain)
Nationalities	Spanish, German

Education

Since 04/2012	PhD Student at the International Leibniz Research School (ILRS) for Microbial and Biomolecular Interactions Jena under the supervision of Prof. Gabriele Diekert.
2012	Diploma in Environmental Sciences, University of Trier (Germany). Diploma thesis: The impact of silver nanoparticles on the activity of antioxidant enzymes in earthworm coelomocytes. Degree: Passed with distinction.
2006	Highschool degree, Instituto de Educación Secundaria Cambrils (Spain).

Professional experience

02 –12/2011	University of Trier: Student lab assistant in the Department of Soil Sciences. Conducting PCR for the DFG project "Genaustausch und Konnektivität von Lumbricidenpopulationen in Abhängigkeit von der Landnutzung".
09 –11/2010	University of Trier: Student lab assistant in the Department of Ecotoxicology. Basic laboratory tasks and supervision of practical courses.
10/2010	Internship at the Center for Histology, Cytology and Molecular Diagnostics Trier, Department of Molecular Pathology (Trier).
02 – 03/2010	Internship at the Center for Histology, Cytology and Molecular Diagnostics Trier, Department of Molecular Pathology (Trier).
09 – 10/2009	Internship at the Fraunhofer Institute for Toxicology and Experimental Medicine, Deparment of Chemical Risk Assessment (Hannover).

Publication record

Mingo FS, Studenik S & Diekert G (2014) Conversion of phenyl methyl ethers by *Desulfitobacterium* spp. and screening for the genes involved. *FEMS Microbiol Ecol* **90:** 783–790.

Mingo FS, Diekert G & Studenik S (2015) Enrichment of *Desulfitobacterium* spp. from forest and grassland soil using the *O*-demethylation of phenyl methyl ethers as growth-selective process. *Microbiology*. Published ahead of print. DOI: 10.1099/mic.0.000218.

Oral presentations at national and international conferences

2013 Studies on the *O*-demethylation potential of *Desulfitobacterium* spp. during the course of lignin degradation.
Felix Sebastian Mingo, Anita Mac Nelly, Sandra Studenik & Gabriele Diekert. 7th ILRS Symposium, Jena (Germany).

2013 Insights into the methylotrophic metabolism of *Desulfitobacterium* spp.
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2014 Abundance of *Desulfitobacterium* spp. in *O*-demethylating anoxic soil microbial communities.
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2014 Abundance of *Desulfitobacterium* spp. in *O*-demethylating anoxic soil microbial communities.
Felix Sebastian Mingo, Anita Mac Nelly, Sandra Studenik & Gabriele Diekert. 7th ILRS Symposium, Jena (Germany).

2015 The role of *Desulfitobacterium* spp. in the global network of *O*-demethylation in soil.
Felix Sebastian Mingo, Sandra Studenik & Gabriele Diekert. 13th Symposium on Bacterial Genetics and Ecology (BAGECO), Milan (Italy).

Poster presentations at national and international conferences

2013 The role of *Desulfitobacterium* spp. in the global network of *O*-demethylation in soil. **Felix Sebastian Mingo**, Sandra Studenik & Gabriele Diekert. Annual Conference of the Association for General and Applied Microbiology (VAAM), Bremen (Germany).

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2015 Anoxic *O*-demethylating microbial communities as a new natural niche for *Desulfitobacterium* spp.
Felix Sebastian Mingo, Anita Mac Nelly, Sandra Studenik & Gabriele Diekert. 8th ILRS Symposium, Jena (Germany).